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GENERATION OF POLYFUNCTIONAL T CELLS AGAINST HCV BY T CELL REDIRECTION AND VACCINATION

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To my beloved Alberto,
this day has finally come

ABSTRACT

The hepatitis C virus (HCV) is the major cause of liver disease and it is estimated that around 170 millions of people are infected worldwide. The available therapy is a combination of pegylated-interferon-alpha, ribavirin and since 2011, also NS3/4A protease inhibitors boceprevir and telaprevir. The standard treatment is associated with considerable side effects and does not cure all patients. Several vaccine candidates, prophylactic and therapeutic, are in the developing phase, but none of them so far have proven to be able to prevent or clear the HCV infection. Thus there is a vital need for an alternative approach for chronically infected HCV patients who do not respond to the standard treatment. Chronic HCV infection leads to severe liver inflammation and subsequent cirrhosis and hepatocarcinoma. T cell failure has been indicated as the main reason of viral persistence. On the contrary, an efficient T cell response has been suggested to hold the key to HCV resolution. In particular, antiviral T cells that are polyfunctional are associated with effective control of HCV replication. The present thesis investigated two different approaches to generate HCV-specific polyfunctional T cells and their potential to reduce HCV RNA+ hepatoma cells and to reduce HCV antigen+ tumor growth was assessed subsequently. Here the two approaches are based on the idea on T cell receptor (TCR) transfer that enables introduction of HCV-antigen specificity from one T cell to another, and DNA vaccination that is enhanced by electroporation. Paper I and II demonstrated that HCV NS3 ($NS3_{1073-1081}$) and NS5A ($NS5A_{1992-2000}$) -specific TCR isolated from HLA-A2 transgenic mice can be transferred to human T cells. Such HCV-specific redirected human T cells demonstrate a different mechanism of action associated with their antigen specificity. NS3-specific TCRs were polyfunctional with potent lytic activity capable to eliminate human hepatoma HCV replicon cells replicating HCV subgenomic RNA, whilst the NS5A-specific TCRs instead ware mainly IFN-γ producers and less cytolytic. This has an interesting implication as the latter may spare the host from unwanted cell injury during elimination of HCV-infected cells. Paper III explored the potential of the NS5A DNA vaccine used in paper II. This pre-clinical study showed that one single injection of the vaccine followed by electroporation could give rise to a polyfunctional T cell response in both wild-type and NS5A-transgenic mice, thought the latter group showed signs of tolerance. A series of truncated NS5A vaccine constructs revealed the locations of the protective antigen that gives the protective immunity. In this study, new murine MHC-I restricted CTL epitope were also identified, which enables immunological studies in HCV transgenic mouse models. These findings provide evidence that high-magnitude and high-quality T cell response able to assist the immune control of HCV can be engineered in vitro and by therapeutic vaccination. It has implications for development of HCV treatments for patients who cannot be cured by antiviral therapy. The TCR-reagents may also serve as tools to gain better understanding of HCV immunology.

LIST OF PUBLICATIONS

I. **Anna Pasetto**, Lars Frelin, Anette Brass, Anila Yasmeen, Sarene Koh, Volker Lohmann, Ralf Bartenschlager, Isabelle Magalhaes, Markus Maeurer, Matti Sällberg and Margaret Chen. Generation of T-cell Receptors Targeting A Genetically Stable and Immunodominant Cytotoxic T-lymphocyte Epitope Within Hepatitis C Virus Non-structural Protein 3.
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II. **Anna Pasetto**, Lars Frelin, Soo Aleman, Fredrik Holmström, Anette Brass, Gustaf Ahlen, Erwin Brenndörfer, Volker Lohmann, Ralf Bartenschlager, Matti Sällberg, Antonio Bertoletti and Margaret Chen. T Cell Receptor-Redirected Human T Cells Inhibit Hepatitis C Virus Replication: Hepatotoxic Potential Is Linked To Antigen Specificity And Functional Avidity.
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III. Fredrik Holmström, **Anna Pasetto**, Veronica Nähr, Anette Brass, Malte Kriegs, Eberhard Hild, Margaret Chen, Gustaf Ahlén and Lars Frelin. A Synthetic Codon-Optimized HCV Non-Structural 5A DNA Vaccine Primes Polyfunctional CD8+ T Cell Responses in Wild-type and NS5A-Transgenic Mice.
Submitted to Journal of Immunology (in revision)

CONTENTS

LIST OF ABBREVIATIONS

aa	amino acid
AIDS	acquired immune deficiency syndrome
ALT	alanine aminotransferase
APC	antigen presenting cell
co	codon optimized
CTL	cytotoxic T lymphocyte
DAA	direct acting antiviral
DC	dendritic cells
DNA	deoxyribonucleic acid
ds	double stranded
ER	endoplasmic reticulum
EP	electroporation
gt	genotype
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immune deficiency virus
HLA	human leukocyte antigen
ID	identification
IFN-α	interferon-alpha
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IL	interleukin
IRES	internal ribosome entry site
NK	natural killer
NS	non-structural
ORF	open reading frame
PDB	Protein Data Bank
pDNA	plasmid DNA
RNA	ribonucleic acid
Ss	single stranded
TCR	T cell receptor
Tg	transgenic
Th	T helper
TNF	tumor necrosis factor
UTR	untranslated region

THESIS SUMMARY

The hepatitis C virus was identified in 1989 by Choo et al (Choo, Kuo et al. 1989) and it is one of the major causes of liver disease since it is estimated that around 170 millions of people in the World are infected (Shepard, Finelli et al. 2005).The available therapy is a combination of pegylated-interferon-alpha, ribavirin and, since their introduction in 2011, also NS3/4A protease inhibitors boceprevir and telaprevir (Jacobson, McHutchison et al. 2011; Poordad and Khungar 2011). This treatment is long, expensive, it leads to several side effects and, most important of all, it does not have a 100% efficacy (Assis and Lim 2012). In particular there is still a group of patients, especially infected with HCV genotype 1, who do not respond to the standard treatment (Ghany, Nelson et al. 2011). Several vaccine candidates, prophylactic and therapeutic, are in the developing phase, but none of them so far demonstrated to be able to prevent or clear the HCV infection (Feinstone, Hu et al. 2012). Therefore the need of an alternative approach, in particular for those patients who do not respond to the standard treatment, is urgent.

The problem with the HCV infection is the chronic persistence of the virus that leads to liver inflammation and subsequent cirrhosis and hepatocarcinoma (Seeff 2002). The main responsible for this failure in clearing the virus is the T cell disfunction that can be caused by several factors like exhaustion, viral escape etc (Thimme, Oldach et al. 2001; Klenerman and Thimme 2012). An efficient T cell response seems to be the key for resolution of the HCV infection and several studies indicate that polyfunctional T cells are superior then monofunctional T cells secreting just one cytokine in fighting viral infection (Iyasere, Tilton et al. 2003; Younes, Yassine-Diab et al. 2003; Zimmerli, Harari et al. 2005; Ciuffreda, Comte et al. 2008).

Therefore we decided to focus our research interest in the generation of polyfunctional T cells against HCV genotype 1 CTL epitopes ($NS3_{1073}$ and $NS5A_{1992}$) associated with viral clearance (Lechner, Wong et al. 2000; Chang, Thimme et al. 2001; Urbani, Uggeri et al. 2001; Shoukry, Grakoui et al. 2003), Chang KM 2001, Urbani S 2001). We used two different approaches: TCR redirection (paper I and II) and DNA vaccination (paper III).

We first generated T-BW cell hybrid clones specific for HCV $NS3_{1073}$ and $NS5A_{1992}$ (paper I). HLA-A2 transgenic mice (Pascolo, Bervas et al. 1997) were vaccinated with DNA plasmids encoding for NS3/4A and NS5A and electroporated to generate HCV-specific CD8+ T cells. The CD8+ T cells from these mice were purified, stimulated with the selected peptides and fused with BW cells. The resulting T-BW hybrid clones were HAT-selected, the survivors were screened for surface CD3 expression and production of IL-2 and IFN-γ after stimulation with peptide loaded T2 cells. In Table S1 the screening process is summarized.

Table S1. Screening summary of T-BW cell hybrid clones.

HCV peptide specificity	Total HAT+ clones	Total CD3+ clones	Stable IL-2+ and IFN-γ+ clones
$NS3_{1073}$	108	95	9
$NS5A_{1992}$	103	87	7

The T-BW hybrid clones were extensively characterized in terms of TCR avidity and affinity with the purpose of identifying the clone with the highest avidity for the specific HCV peptide. The final aim of this process is indeed the identification of TCR genes for TCR redirection of human T cells and it has been shown that high avidity TCR have a better antiviral action (Appay and Iglesias 2011). We chose to use murine TCRs because: 1) they have a higher surface expression in human cells compared to the endogenous TCR (Cohen, Zhao et al. 2006); 2) they do not pair with the human alpha and beta TCR chain to circumvent TCR mispairing that can lead to off target toxicity (Kieback and Uckert 2010); 3) clinical adoptive transfer studies with TCR redirected T cells expressing murine TCRs have already demonstrated that the immune response against these exogenous TCRs do not affect the outcome of the therapy (Davis, Theoret et al. 2010).

As result of the screening process two TCR candidates for each HCV CTL peptide were chosen for the TCR redirection study (paper II). For $NS3_{1073}$ the clones H4 and F8 demonstrated the highest avidity while clones 69 and 19 showed the highest avidity for $NS5A_{1992}$ (in Table S2 the CDR3 loops from the TCRs chosen for the T cells redirection study are summarized). These TCR genes were codon optimized for expression in human cells and cloned into a retroviral vector. The MP71 vector was chosen because it is modified to guarantee higher transgene expression in T cells (Engels, Cam et al. 2003; Leisegang, Engels et al. 2008). The structure of the gene cassette was designed to ensure an even expression of alpha and beta TCR chains so the two genes were linked by the autoprotease 2A sequence in a single construct. Human PBL from healthy donor and HCV chronic patients were transduced by spinoculation and the TCR expression and functionality were evaluated by flow-cytometry and functional analyses.

Table S2. CDR3 loops from alpha (A) and beta (B) chains of the TCRs selected for the T cell redirection study.

A)

TCR ID	CDR3 alpha chain
H4	CAMREITGNTGKLIFGL
F8	CAVSNMGYKLTFGT
19	CAASLITGNTGKLIFGL
69	CIVTDLGITGNTGKLIFGL

B)

TCR ID	CDR3 beta chain
H4	CASSDALGGEDAEQFFGPGTRL
F8	CASSQEMGGALEQYFGPGTRL
19	CASSLTANTEVFFGKGTRLT
69	CASGDEGYNSPLYFAAGTRLT

The most interesting finding was the correlation between the antigen specificity and the antiviral activity. We demonstrated that while NS3-specific T cells present a polyfunctional profile with a clear lytic activity against human hepatoma HCV replicon cells, NS5A-specific T cells mainly secrete IFN-γ and interfere with viral replication in

a less effective manner and without lytic activity. Our result is in accordance with other studies were HCV replicon models were used to show that the non-cytolytic mechanism contributes to control HCV infection (Lohmann, Korner et al. 1999; Blight, Kolykhalov et al. 2000) and in particular IFN-γ is the main inhibitor of viral replication (Frese, Schwarzle et al. 2002; Jo, Aichele et al. 2009). *In vivo* studies conducted with chimpanzees also showed that viral clearance can occur in the absence of elevated alanine aminotransferase levels and with only minimal evidence of liver cell injury but with detectable IFN-γ messenger RNA in the liver (Bigger, Brasky et al. 2001; Su, Pezacki et al. 2002; Thimme, Bukh et al. 2002). Taken together these findings point out the possibility of eliminating HCV replication from infected cells without actual killing. This is particularly important for a possible therapeutic use of HCV redirected T cells in chronic patients who already suffer from a severe liver damage. This approach anyway would be intended as a complement to the antiviral therapy and moreover, the combined effect of NS3 and NS5A redirected T cells still needs to be evaluated *in vivo*.

The second strategy we applied to generate HCV-specific polyfunctional T cells was DNA vaccination followed by electroporation. Previous studies by Ahlén et al (Ahlen, Soderholm et al. 2007) showed that a specific and effective CTL response against NS3/4A could be generated by using this method. The advantages of this vaccination procedure in contrast to a simple DNA injection are the higher DNA uptake and the increased immune stimulation caused by the electroporation itself (adjuvant effect) (Mathiesen 1999; Gronevik, Mathiesen et al. 2005). In paper III we focus our interest on NS5A because it is an essential protein in the HCV replication machinery (Blight, Kolykhalov et al. 2000; Tellinghuisen, Marcotrigiano et al. 2005) and it has also been proposed as a target for antiviral therapy (Coelmont, Hanoulle et al. 2010; Nettles, Gao et al. 2011; Lawitz, Gruener et al. 2012). Therefore we wanted to evaluate the immunological response to HCV NS5A after DNA vaccination in wild-type and NS5A transgenic mice and to do so we also identified novel murine CTL epitopes.

The most interesting finding was that the NS5A-specific immune response could be raised in both wild-type and transgenic mice even if the second group showed evidences of tolerance (with lower levels of cytokine production after immunization). The two groups of mice were also challenged with tumor cells expressing NS5A after vaccination and an effective protection against tumor growth was found. In accordance with other vaccination studies were polyfunctional T cells are associated with an efficient immune response (Aagaard, Hoang et al. 2009; Burgers, Riou et al. 2009; Abel, Tameris et al. 2010; Derrick, Yabe et al. 2011; Lang Kuhs, Ginsberg et al. 2012; Tan, Eriksson et al. 2012) our findings highlight the validity of NS5A DNA vaccination since it was possible to generate a polyfunctional T cell response even in a tolerized mouse model.

In conclusion we have successfully obtained polyfunctional T cells directed against NS3 and NS5A proteins by using two distinct approaches: TCR redirection and vaccination. This has implications for development of HCV treatments for patients who cannot be cured with the current antiviral therapy. The TCR-reagents may also serve as tools to gain better understanding of HCV immunology.

1 INTRODUCTION TO HEPATITIS C VIRUS INFECTION

When people think about a worldwide viral infection starting in the early 80s, the first thought usually goes to the acquired immune deficiency syndrome (AIDS) and to the human immune deficiency virus (HIV). However, during the same period another blood-borne virus was also spreading silently and undetected and, like HIV this infectious agent was mostly targeting drug users and blood transfusion recipients. For several years, even for decades, most of the infected people did not notice any discomfort, not until the late disease stages when the real nature of the virus was already evident with a full attack to the liver often causing cirrhosis and cancer. This virus was identified and named hepatitis C virus in 1989 (Choo, Kuo et al. 1989). Since then several million people were diagnosed for hepatitis C virus infection and nowadays it is estimated that around 170 million people are infected and of these 130 million are chronically infected and at risk for cirrhosis and liver cancer (Shepard, Finelli et al. 2005). In the Unites States, Europe and Japan the rate of transmission is currently decreasing thanks to disposable medical instruments and blood screening supply for the early diagnosis, but in developing countries the infection is spreading tremendously (John-Baptiste, Yeung et al. 2012). The traditional alpha-interferon based antiviral treatment has been long, expensive and cause of numerous side effects, besides, it was effective in only 50 to 80% of the cases depending on the viral genotype (John-Baptiste, Yeung et al. 2012). Recently two NS3/4A protease inhibitors, a first generation of direct acting antiviral (DAA) drugs, have been introduced in combination with pegylated interferon alpha and ribavirin for the treatment of patients infected with HCV genotype 1 with promising results (Sarrazin, Hezode et al. 2012). Despite this the increasing number of chronic hepatitis C cases is turning this disease into a very intense public health issue; therefore the need for a better treatment or therapeutic vaccine remains a major challenge for public health.

1.1 THE HEPATITIS C VIRUS (HCV)

The hepatitis C virus (HCV) is an enveloped single-stranded RNA virus belonging to the *Flaviviridae* family (Robertson, Myers et al. 1998). The genome is around 9.6 kb encoding for a long polyprotein which is then cleaved by both host and viral factors into different structural and non-structural proteins. The virion consists of an outer envelope composed of the structural E1 and E2 proteins covering the nucleocapsid (core) containing the RNA genome. In addition, several non-structural proteins are required for viral replication and packaging of the viral genome into the capsid (p7, NS2, NS3, NS4A, NS5A and NS5B) (Figure 1) (Kato, Yokosuka et al. 1990; Choo, Richman et al. 1991).

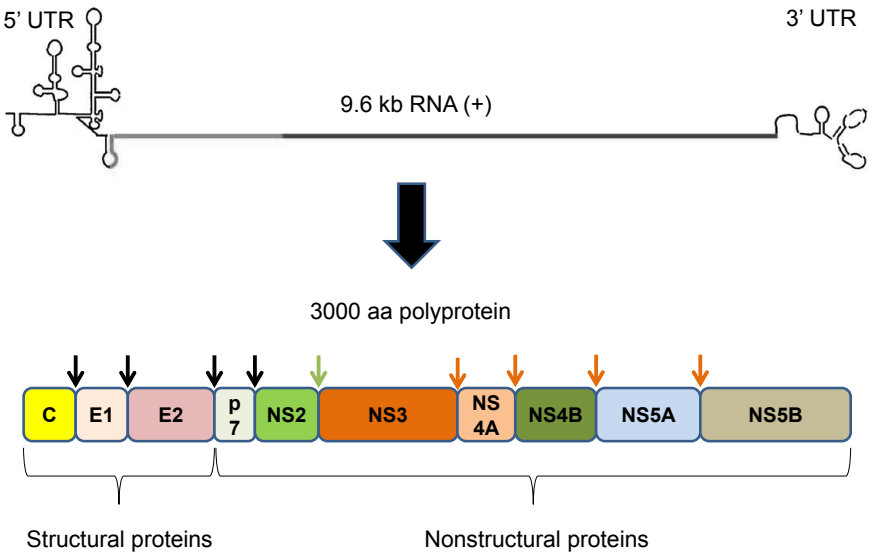

Figure 1. HCV genome and polyprotein. The HCV genome is a 9.6 kb RNA molecule of plus strand polarity. The genome is translated into a 3000 amino acid polyprotein that is then cleaved by cellular peptidases (black arrows) and viral peptidases (NS2 green arrow and NS3 orange arrows) into three structural proteins (core, E1 and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B). Modified from Georgel et al (Georgel, Schuster et al. 2010).

The HCV genome is characterized by a high level of heterogeneity that allowed classification of 6 different viral genotypes (indicated with numbers from 1 to 6) differing of around 32% in their genome plus several subtypes (indicated with letters a, b, c etc.) differing of around 22% at the nucleotide level (Simmonds 1994).

1.2 EPIDEMIOLOGY

The HCV genotypes are differently distributed worldwide. Genotype 1 is for example the most prevalent genotype in Europe (Figure 2).

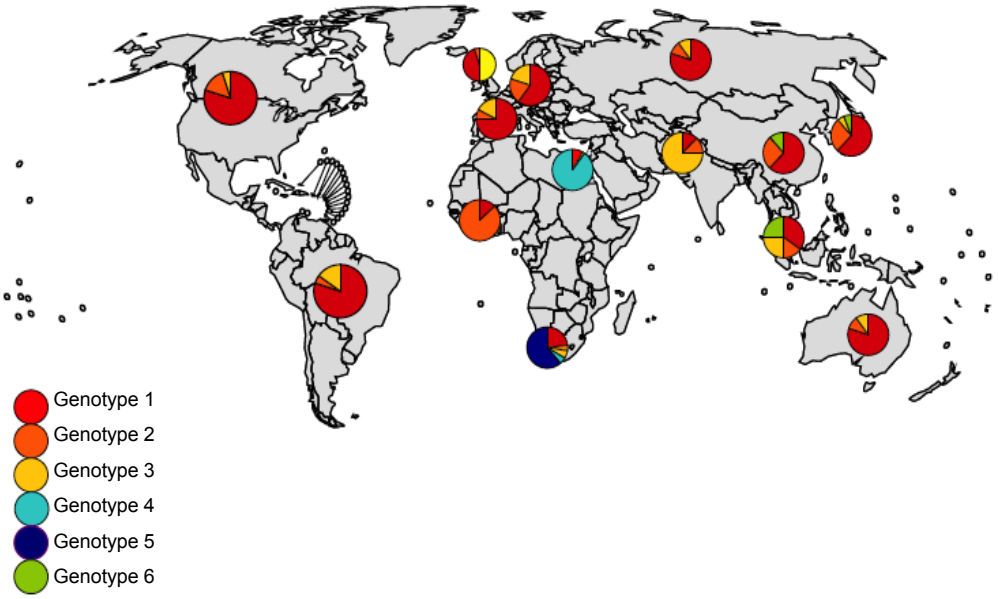

Figure 2. HCV genotype prevalence (World Health Organization 2009).

Being infected with different HCV genotypes implies different chances of virus clearance. Patients infected with genotype 2 and 3 are in fact curable with the standard care therapy (pegylated interferon-alpha in combination with ribavirin) in around 80-90% of the cases, while patients infected with genotype 1 are cured only in around 40% of the cases (Fried, Shiffman et al. 2002; Patel and McHutchison 2004). Recently, the introduction of new antiviral drugs (two first generation NS3/4A protease inhibitors) for genotype 1 infected patients has improved substantially the cure rate for this group (Jacobson, McHutchison et al. 2011; Poordad and Khungar 2011). A second generation of DAAs is currently in preclinical or clinical development stage with drugs directed against several viral targets including NS3/4A, NS5B polymerase and NS5A (Sarrazin, Hezode et al. 2012).

1.3 HCV LIFE CYCLE AND IMMUNE EVASION

The route of infection, as previously mentioned, is basically blood-blood contact with the most affected individuals being, in the western developed world, intravenous drug users. However, a minor route of transmission is also represented by vertical transmission (mother-child), sexual exposure and other types of contacts with infected blood (tattoo etc) (World Health Organization Hepatitis C fact sheet n. 164, July 2012). After entering the blood stream the virus targets primarily the hepatocytes in the liver. To enter a cell, the envelope proteins E1 and E2 must bind to specific cellular receptors and so far it has been shown that the combination of at least four host molecules is needed: CD81 (Pileri, Uematsu et al. 1998), scavenger receptor type B class I (SCARB1) (Scarselli, Ansuini et al. 2002), claudin 1 (CLDN1) (Evans, von Hahn et al. 2007) and occludin (OCLN) (Ploss, Evans et al. 2009). After the virion has attached to the cell surface, it is able to enter via clathrin-mediated endocytosis (Blanchard, Belouzard et al. 2006) while the release of the capsid into the cytosol is mediated by lowering the pH in the endocytes (Hsu, Zhang et al. 2003; Bressanelli, Stiasny et al. 2004). Once inside the cell, the RNA+ HCV genome is translated into a long polyprotein via the IRES sequence located at the 5`UTR. As previously mentioned, this polyprotein is then cleaved by both host and viral proteases into ten proteins, of which one of the most important for immune evasion is the NS3/4A (Figure 3A-C). Another non-structural protein important for this purpose is NS5A, which is constituted by tree domains (I, II, and III) associated with different functions (Figure 3D).

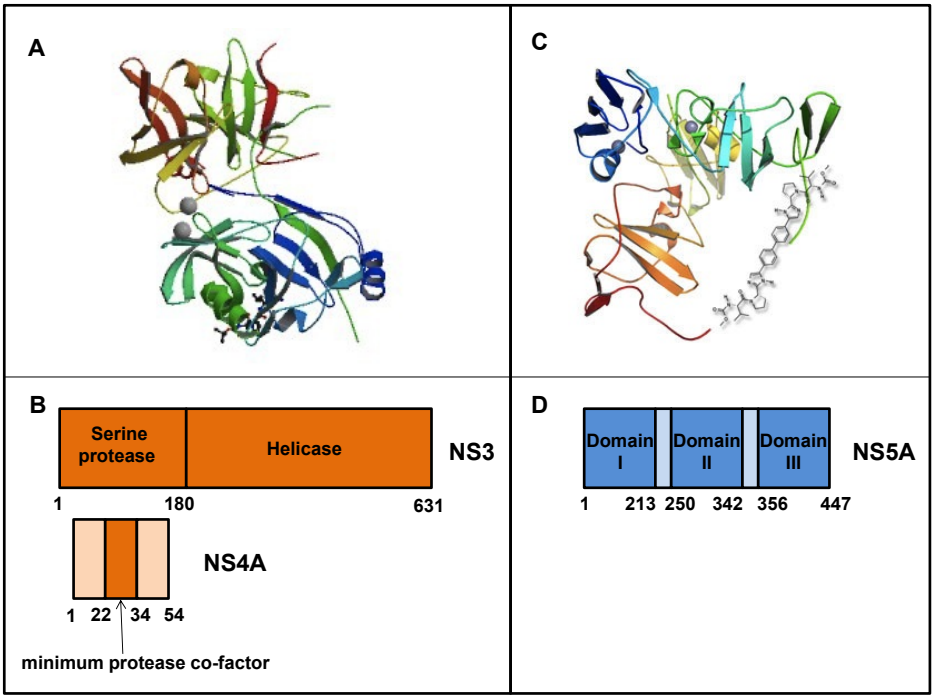

Figure 3. (A) NS3/4A crystal structure (Chen, Njoroge et al. 2005) (PDB ID 2A4Q). (B) NS3 and NS4A schematic representation: NS3 is a 631 amino acid protein with two domains: serine protease domain (1-180), which cleaves the HCV polyprotein sites, and a RNA helicase domain (180-631): NS4A is a 54 amino acid protein that stabilizes NS3 anchoring to the cytoplasmic side of the endoplasmic reticulum. The amino acids 22-34 are the minimum domain for the serine protease co-factor activity (Bartenschlager, Ahlborn-Laake et al. 1993; Kwong, Kim et al. 2000). (C) NS5A crystal structure zinc finger in domain I (Tellinghuisen, Marcotrigiano et al. 2005) (PDB ID 1ZH1). (D) NS5A schematic presentation. Domain I (1-213) is a membrane anchoring domain responsible for the NS5A dimerization (Hwang, Huang et al. 2010) and essential for RNA replication; domain II (250-342) contains the elements responsible for the interference with IFN signaling; domain III (356-447) is involved in the assembly and production of HCV particles (Gale, Blakely et al. 1998; Appel, Zayas et al. 2008; Tellinghuisen, Foss et al. 2008).

The NS3/4A complex has been shown to target at least three different host anti-viral elements: the mitochondrial antiviral signaling protein (MAVS), the TIR domain-containing adaptor inducing IFN-β (TRIF) and the T cell protein tyrosine phosphatase (TCPTP) (Brenndorfer, Karthe et al. 2009; Morikawa, Lange et al. 2011) (Figure 4). NS5A domain I is a membrane-anchoring domain that seems to be essential for RNA replication (Tellinghuisen, Foss et al. 2008); domain II was suggested to interfere with the interferon signaling pathways through interferon-dependent induced protein kinase R (PKR) (Gale, Blakely et al. 1998; Appel, Schaller et al. 2006) and also to impair HCV-specific T cell response in the liver (Kriegs, Burckstummer et al. 2009) while domain III seems to be connected to the assembly and production of novel viral particles (Appel, Zayas et al. 2008).
The mechanism of virion assembly can be divided in two steps: early assembly located in the cytosolic side of the ER where core, NS5A and NS3 form immature non-infectious virions; late assembly that consists in the acquisition of the lipid envelope

together with E1 and E2; NS2 seems to mediate the interaction between E1, E2 and the immature virions conferring infectivity (Jones and McLauchlan 2010).

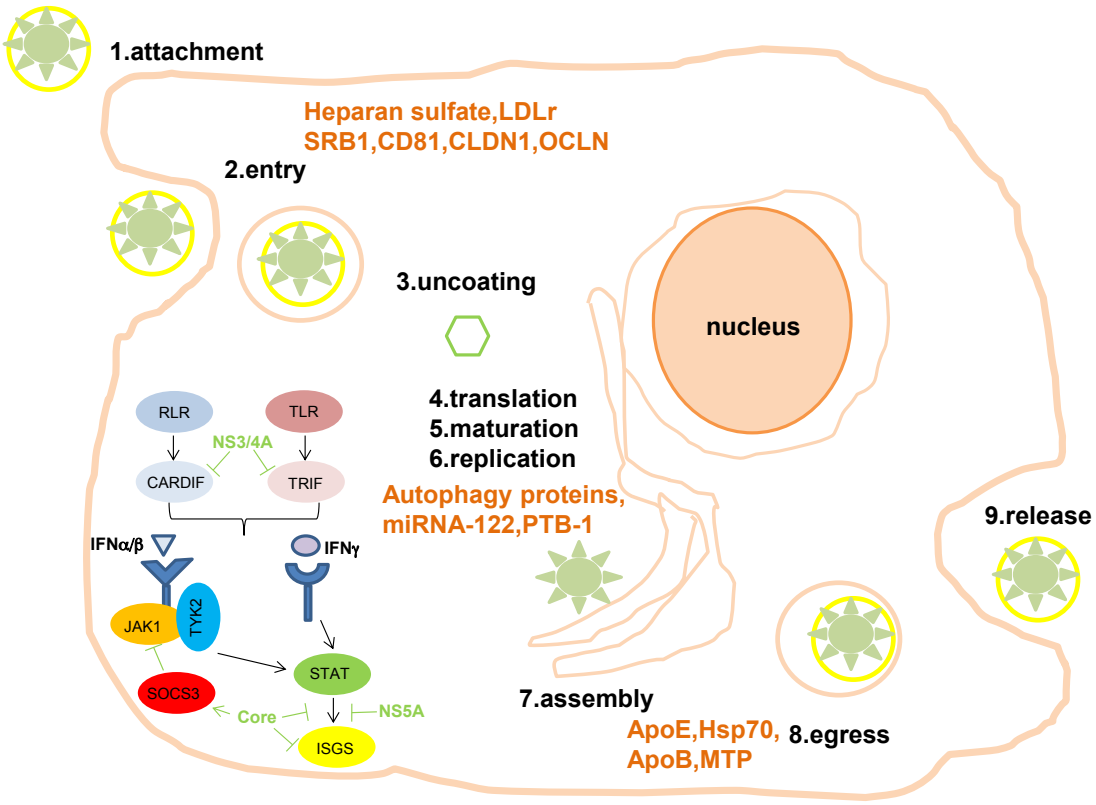

Figure 4. Interaction of HCV and the host cell. During the HCV replication cycle many host cell factors are involved (red). Heparan sulfate, low-density lipoprotein receptor (LDLr), scavenger receptor B1 (SR-B1), CD81, claudin 1 (CLDN1) and occludin (OCLD) are involved for the attaching and entry steps; autophagy proteins, miRNA-122 and polypyrimidine tract binding protein 1 (PTB-1) are involved in the translation, maturation and replication steps; ApoE, ApoB, heat shock protein 70 (Hsp70) e microsomal transfer protein (MTP) are involved in the assembly and egression steps. Core, NS3 and NS5A HCV proteins also interact with the IFN signaling pathways. Modified from Georgel et al (Georgel, Schuster et al. 2010).

1.4 CLINICAL FEATURES

The infection with HCV can results in two different outcomes: an acute infection which is mostly followed by spontaneous viral clearance (about 25% of cases) or, if viremia persists for more than six months, a chronic infection (about 75% of the cases). Several factors have been associated with viral clearance including age (less than 40 years old) (Micallef, Kaldor et al. 2006), female gender (Bakr, Rekacewicz et al. 2006), disease presented with lower viral load (Villano, Vlahov et al. 1999) and sustained T cell responses against nonstructural proteins (Ward, Lauer et al. 2002; Rehermann 2009).
In the majority of cases however, the disease becomes chronic with persistently detectable viral load and anti-HCV antibodies. The liver is affected by a gradual

inflammatory damage followed by progressive fibrosis that can lead to cirrhosis and hepatocarcinoma.

1.5 LIVER TOLERANCE AND T CELL IMMUNITY

In the case of HCV infection, the first site of antigen presentation to the lymphocytes is particularly important as it is believed that the unique features of the liver would induce immune response into tolerance (and then chronic infection) rather than immunity against the invading pathogen (Crispe 2009) as that several cell types in liver have tolerogenic potentials (see below).

1.5.1 Liver tolerance

It has been calculated that approximately 75% of the blood present in the liver comes directly from the gut through the portal vein; therefore being loaded with food and bacterial antigens (Bowen, McCaughan et al. 2005). This would be the main reason for making the liver milieu more prone to drive immune cells towards tolerance instead of immune response (Crispe 2009). In line with this, the local and systemic tolerance effect has also been attributed to specialized liver resident cells expressing anti-inflammatory mediators and inhibitory cell surface ligands for T cell activation (Figure 5). In the subsequent paragraph these different antigen presenting cell types will be discussed.

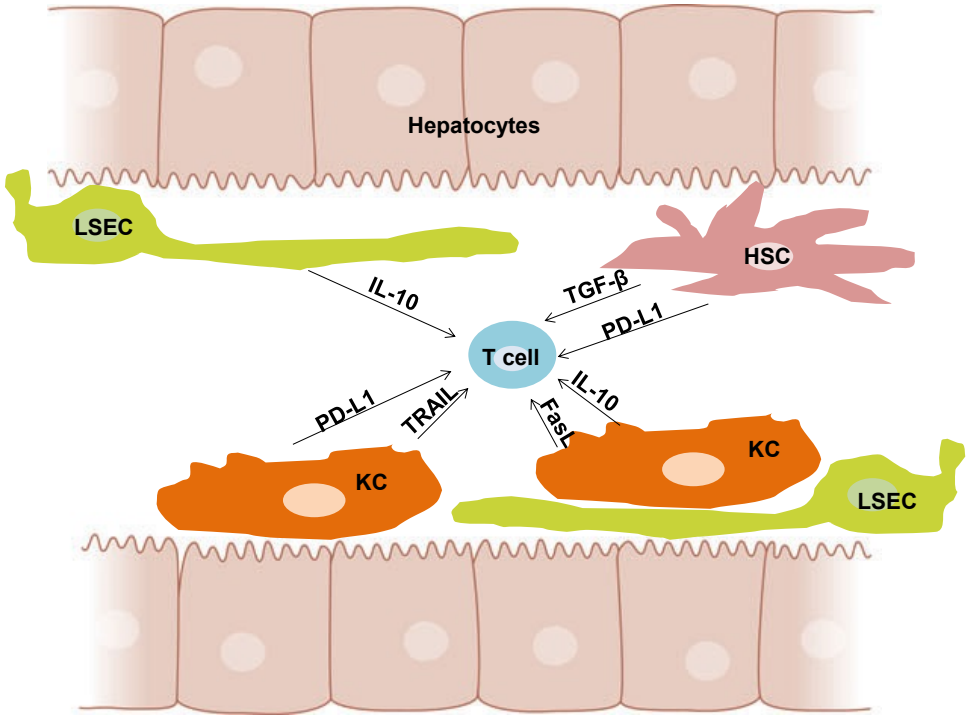

Figure 5. Possible mechanisms of T cell tolerance in the liver. Liver sinusoidal endothelial cells (LSEC) express adhesion molecules that facilitate the trapping of activated T cells, Kupffer cells (KC) express also TRAIL and Fas ligand (FasL) which make T cells undergo apoptosis or be phagocytosed. In addition, T cells are also exposed to immunosuppressive cytokines, like IL-10 and TGF-β1 and to inhibitory ligands like PD-L1 coming also from hepatic stellate cells (HSC). Modified from Crispe et al (Crispe 2009).

Conventional antigen presenting cells (APCs). These are dendritic cells (DCs), which take up the antigens at the site of the infection, the liver in this case, and then migrate to the lymph nodes in order to present the antigen to specific T cells. DCs isolated from liver tissue express very low levels of class II major histocompatibility complex (MHC) molecules and co-stimulatory molecules (CD40, CD80, CD86) exhibiting an immature phenotype (Racanelli and Rehermann 2006; Selmi, Mackay et al. 2007; Crispe 2009). Accordingly, liver DCs produce preferentially IL-10 instead of Th1 activating cytokines thereby promoting a Th2 cytokine response. This feature also seems to favor CD4+CD25+Foxp3+ regulatory T cells (Goddard, Youster et al. 2004; Bamboat, Stableford et al. 2009).

Liver sinusoidal endothelial cells (LSECs). These cells constitutively express MHC class I and II as well as co-stimulatory molecules like CD40, CD80 and CD86. They are able to take up antigens and present them to CD4+ and CD8+ T cells. The induction of tolerance correlated also with the induction of negative co-stimulatory molecule PD-L1 by LSECs (Knolle, Uhrig et al. 1998; Limmer, Ohl et al. 2000; Knolle and Limmer 2001; Limmer, Ohl et al. 2005).

Kupffer cells (KCs). These are tissue resident macrophages able to release pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α thus promoting infiltration of neutrophilic granulocytes involved in elimination of bacteria (Knolle, Lohr et al. 1995). TNF-α is a cytotoxic factor in the liver because it leads hepatocytes to apoptosis under pathological conditions (Schumann and Tiegs 1999; Wullaert, van Loo et al. 2007). On the contrary, a low concentration of TNF-α makes hepatocytes resistant to apoptosis (Sass, Shembade et al. 2007; Haimerl, Erhardt et al. 2009). KCs also produce IL-2 and IL-18. These cytokines, among others, activate NK cells to produce antiviral IFN-γ. However after this initial production of pro-inflammatory cytokines KCs release IL-10 which downregulates the production of TNF-α, IL-6 and other cytokines (Knolle, Lohr et al. 1995) and probably contribute to induce tolerance.

Hepatic stellate cells (HSCs). HSCs have mainly been described for their participation in hepatic fibrosis and storage of vitamin A. However, they have also been shown to function as APCs and to be able to present proteins or lipid antigens to CD8+, CD4+ and NKT cells (Winau, Quack et al. 2008). Because of their ability to store vitamin A and to produce TGF-β in response to inflammation and injury they may also exhibit tolerogenic functions. CD4+ T cells can in fact be converted into induced regulatory T cells by vitamin A derived retinoic acid and/or TGF-β (Strober 2008). Moreover activated HSCs express PD-L1 (Yu, Chen et al. 2004).

Hepatocytes. Hepatocytes mainly perform metabolic functions. However, they also seem to participate to the immunoregulation in the liver by their ability as APCs. It has been shown that PD-L1 is induced in hepatocytes by viral infection as well as by type I and type II interferons (Muhlbauer, Fleck et al. 2006) together with IL-10 that, as also mentioned previously, is the dominant cytokine produced by resident DCs, KCs and LSEC.

1.5.2 T cells in HCV infection

In general the CD8+ cytotoxic T lymphocytes (CTL) are essential for an effective immune response to all viral infections (McMichael and Hanke 2003). Viral replication is mainly suppressed by CTLs in three different ways: 1) production of IFN-γ; 2) lysis of infected cells via Fas-FasL interaction and 3) lysis of target cells by delivery of granzyme and perforin (this third mechanism is the most rapid and usually the most important in anti-viral defense). An effective antiviral CTL response is defined as a response that reduces either the viral load or the incidence or prevalence of virus-associated disease (Bangham 2009). In the following paragraph the role of T cells during HCV infection will be discussed.

Both virus specific CD4+ and CD8+ T cell populations are determinant for the outcome of HCV infection. CD4+ T cell help is in fact required for a successful CD8+ CTL cell mediated viral clearance (Neumann-Haefelin, Spangenberg et al. 2007). As previously mentioned, viral replication can be suppressed by lysis of target cells by specific CTLs, but in the case of HCV, a non-cytolytic mechanism of viral clearance would be preferable as infected cells being cured instead of killed would result in less hepatotoxic effects (Guidotti and Chisari 2006). In the early phase of acute HCV infection (4-8 weeks after infection) it is possible to detect a vigorous and specific CD8+ T cell response against several viral epitopes (Gruner, Gerlach et al. 2000; Thimme, Oldach et al. 2001). These virus specific CD8+ T cells present a so-called "stunned phenotype" because they are not able to secrete antiviral cytokines like IFN-γ (Lechner, Wong et al. 2000; Thimme, Oldach et al. 2001). In a subsequent phase of infection these stunned cells gain back their capacity to secrete antiviral cytokine and this is associated with decline of viremia and, in the resolving infection, with viral clearance (Neumann-Haefelin, Spangenberg et al. 2007). However in the chronic phase of HCV infection, the HCV-specific T cells appear impaired in mainly two ways: by T cell exhaustion and viral escape. T cell exhaustion is defined by impaired CD8+ T cell effector functions and characterized by co-expression of several inhibitory receptors such as PD-1, 2B4 and CD160 while viral escape is not a universal mechanism. It is in fact limited by fitness cost, for example by the inability to tolerate mutations within highly constrained epitopes (Klenerman and Thimme 2012).

1.5.3 Intrahepatic lymphocytes

T cells. Several studies on experimentally infected chimpanzees have shown that a strong HCV-specific T cell response inside the liver is important for reduction of viral load and clearance of acute infection (Weiner, Erickson et al. 1995; Cooper, Erickson et al. 1999; Thimme, Bukh et al. 2002; Shoukry, Grakoui et al. 2003). However, in spite that intrahepatic HCV-specific CD8+ T cells are present in the majority of patients with chronic HCV infection, it is clear that a large fraction of these CD8+ T cells are impaired especially in their ability to secrete IFN-γ (Spangenberg, Viazov et al. 2005; Neumann-Haefelin, Timm et al. 2008). A subset of HCV-specific intrahepatic CD8+ T cells can also secrete IL-17, and was found to have a possible association with low grade of liver inflammation (Grafmueller, Billerbeck et al. 2012). Interestingly this subset shows a different antigen-specificity compared with IFN-γ producing CD8+ T cells, and whether they exhibit antiviral mechanisms remain to be determined. The

localization of CD8+ T cells within the liver is required for termination of HCV replication while the control of viremia is dependent on a rapid and massive expansion of CTLs (Shoukry, Grakoui et al. 2003). A strong intrahepatic T cell response is critical to clear the infection but it was estimated that around 70% of the infected individuals fail to mount this response and become chronic (Bowen, McCaughan et al. 2005). Major factors contributing to the intrahepatic T cell failure are likely to be the liver tolerance effect as we previously discussed and the continue rise of viral escape mutations.

NK and NKT cells. The liver also has an unusually high frequency of natural killer (NK) cells and NK T (NKT) cells. In patients with chronic HCV infection, NK cells are reduced in their frequency and functionality. The intrahepatic NK cells in particular show dysfunctional features with a reduced TRAIL and CD107a expression, indicating the existence of a lytic defect in the NK-cells (Varchetta, Mele et al. 2012). Instead, IL-10 and TGF-β are produced resulting in production of Th2 cells and Tregs. The NKT cells are also highly abundant in the liver constituting up to 50% of intrahepatic lymphocytes. They represent a unique subset of T lymphocytes that have TCR and NK markers. A decrease in intrahepatic NKT cells has also been reported in chronic HCV patients. NKT cells have a role in the deleterious effects mediated by immune cells during chronic liver inflammation, as the numbers of activated NKT cells have been found to correlate with the degree of hepatocellular damage and onset of fibrosis (Nuti, Rosa et al. 1998; de Lalla, Galli et al. 2004).

2 ANTIVIRAL THERAPY

Pegylated interferon-alpha and ribavirin have constituted as the standard of care (SOC) of chronic HCV infection during the past decade. SOC therapy is often long and causes considerable side effects including fewer, fatigue, myalgia anemia, depression, skin rash and gastrointestinal symptoms. This combination had a synergistic antiviral effect but, depending on the HCV genotype, it was efficient in only 33%-42% of patients with genotype 1 and in 90% of those with genotypes 2 and 3 (Fried, Shiffman et al. 2002). In 2011, two protease inhibitors (telaprevir and boceprevir) were approved for use in combination with SOC therapy for genotype 1 patients and further improved the treatment efficiency to around 70% (Jacobson, McHutchison et al. 2011; Poordad and Khungar 2011). As for HIV, it is likely that, due to the pre-existing viral mutants and the high mutation rate of the HCV genome, drug resistance will eventually emerge during treatment with specific viral inhibitors; therefore it has been anticipated that a combination of drugs acting in different stages of the viral life cycle needed to be developed (Figure 6). In addition to these two first-generation NS3/4A inhibitors, there is now a large number of new HCV inhibitors in clinical development, which are discussed below.

Other NS3/4A inhibitors with better pharmacokinetic and tolerability than telaprevir and boceprevir have been developed. TMC435 (Tibotec/Janssen-Cilag) and BI201335 (Boehringer-Ingelheim) for example, showed high rates of rapid virological response, together with sustained virological response rates of the same order or higher than those reported with telaprevir and boceprevir (Fried, Hadziyannis et al. 2011; Gane, Rouzier et al. 2011). These drugs are now evaluated in phase III clinical trials in combination with pegylated IFN-α and ribavirin (Sarrazin, Hezode et al. 2012).

Nucleoside/nucleotide analogue inhibitors of the HCV RNA-dependent RNA polymerase. These molecules function as false substrate for the polymerase so their incorporation in the newly synthetized RNA actually results in the termination of the replication process (Sarrazin, Hezode et al. 2012). Though they have a low "genetic barrier" to resistance, i.e. single amino acid substitutions are able to confer drug resistance *in vitro*, the resistant virus variants are poorly fit, thus may be considered as high "barrier" resistance DAAs.

Non-nucleoside inhibitors of the HCV RNA-dependent RNA polymerase. The structure of the RNA-dependent RNA polymerase can be compared to the shape of a right hand. The non-nucleoside inhibitors create a steric bulk for the polymerase binding to one of the four allosteric sites at the surface of the protein. Inhibitors binding the "thumb domain I" or the "palm domain I" are designated BI207127 (Beaulieu, Jolicoeur et al. 2010), VCH-759 (Cooper, Lawitz et al. 2009), and ABT-333, ABT-072 (Abbott) (Sarrazin, Hezode et al. 2012) and Tegobuvir (GS-9190) Gilead (Shih, Vliegen et al. 2011) respectively. All these drugs are active against HCV genotype 1. For all the molecules resistant viral mutants have been found (Sarrazin, Hezode et al. 2012).

NS5A inhibitors. The first inhibitor tested in clinical trials was Daclatasvir (BMS-790052), which binds the protein domain I and is specific for HCV genotype 1 (Gao,

Nettles et al. 2010). This drug is currently in phase II clinical trials in combination with pegylated interferon-alpha and ribavirin, or in interferon-free trials with NS3/4A protease inhibitors or with nucleotide analogues (Sarrazin, Hezode et al. 2012). Other NS5A inhibitors in developing phase are BMS-824393 (Bristol-Myers Squibb), AZD7295 (Arrow Therapeutics/AstraZeneca), PPI-461 (Presidio) and GS-5885 (Gilead) (Gane, Roberts et al. 2010; Gao, Nettles et al. 2010; Nettles, Gao et al. 2011).

Host-targeting agents (HTA). HTAs are novel antiviral agents that target various host cell factors required during the HCV life cycle. The strengths with HTA are high genetic barrier to resistance, the pan-genotypic antiviral activity, and possible complementary mechanisms of action with DAAs. Currently tested in clinical trials are inhibitors against host cellular target such as SR-BI (ITX5061), miR122 (Miravirsen), HMGCoA reudctase (Statins), Cyclophilin A (SC-635 and Alisporivir) and Glucosidase (MX-3253) (Zeisel, Lupberger et al. 2012). So far with cyclophlin inhibitors no resistant viral mutants have been identified (Flisiak, Feinman et al. 2009).

Although DAAs increase the response to IFN-based anti-HCV therapy, they also lead to selection of drug-resistant variants. Given the reported side effects and potential drug-drug interactions, anti-HCV DAAs are not approved for several groups of patients including those undergoing liver transplantation, immunosuppressed or HCV/HIV co-infected (Sarrazin, Hezode et al. 2012). Thus, in spite that early clinical trials have shown excellent outcomes for DAA combinations for treating HCV patients, novel antivirals for the difficult-to-treat patients need to be developed.

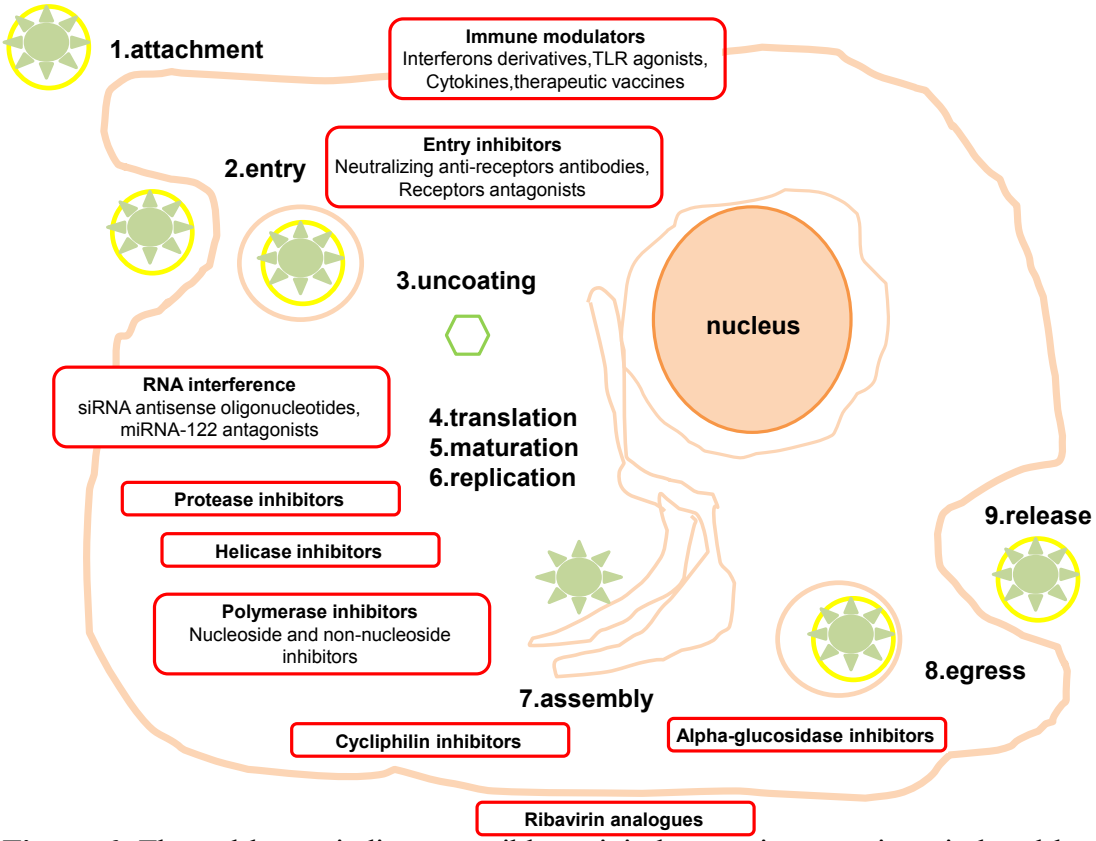

Figure 6. The red boxes indicate possible antiviral strategies targeting viral and host factors mediating HCV infection. Immune modulators and ribavirin analogs are shown outside the cell because they target multiple pathways. Modified from Georgel et al (Georgel, Schuster et al. 2010).

3 VACCINE

The purpose of vaccination is to elicit a strong, specific and effective immune response that can be acquired by the host as immunological memory and be recalled rapidly when the pathogen is re-encountered. Generating a strong CTL response is important for the resolution of viral infections. In the case of chronic diseases such as HCV, the vaccine can be intended also as therapeutic to boost or redirect the already existing but not optimal antiviral immunity of the host. Developing an HCV vaccine with an existing therapy and in parallel to the introduction of new antiviral drugs like Telaprevir and Boceprevir, can appear dispensable. However in this respect, one must keep in mind the current treatments are not efficient in all patients and are accompanied with very high cost and adverse effects (Tungol, Rademacher et al. 2011). The current state of HCV vaccines in clinical trials, both prophylactic and therapeutic, is discussed here.

Prophylactic vaccine. The aim here is to prevent the infection thus avoiding completely the need of a costly and unpleasant therapy. The ideal mechanism of action would basically be to induce a B cell response with effective neutralizing antibodies in parallel to the stimulation of both T helper cells and CTLs. Clinical studies on prophylactic vaccines are thus often designed to induce T-cell responses that target the non-structural proteins of HCV, or antibody responses that target the E1E2 envelope proteins (Feinstone, Hu et al. 2012). The idea is that elimination of persistent infection will prevent chronic liver disease, which is the major problem with HCV infection (Seeff 2002). Ongoing and completed clinical studies are the vaccines developed by Chiron/Novartis and Okairos. Purified recombinant viral envelope protein vaccine with Chiron´s MF59 adjuvant showed promising results in the preclinical and phase I clinical trials, with high levels of neutralizing antibodies and a strong T helper response (Frey, Houghton et al. 2010; Houghton 2011). Adenovirus- or MVA vector-delivered NS3 or NS3-NS5B vaccines conducted by Okairos or NIAID also represent interesting prophylactic vaccine candidates. Using new adenoviral vectors based on rare serotypes [human adenovirus 6 (Ad6) and chimpanzee adenovirus 3 (ChAd3)] expressing NS proteins from HCV genotype 1b, Okairos Ad6NSmut/AdCh3NS3mut vaccine succeeded to generated a broad T cell response and the vaccine is going to be tested in a phase II clinical trial (Barnes, Folgori et al. 2012). An important issue is to define functional markers of protection for a vaccine candidate and these markers need to be evaluated during the developing phase, such markers can be antibody epitopes, T-cell phenotypes, homing profiles, central and effector memory T-cell phenotypes, T-helper function and proliferation (Feinstone, Hu et al. 2012). As exemplified by the HIV vaccine research, the experience is that if a T cell vaccine candidate fails to clear the virus the major reason is that the virus specific CD8+ T cells are secreting just one cytokines, mostly IFN-γ, therefore it is desirable to generate a polyfunctional T cell response instead (Harari, Bart et al. 2008).

Therapeutic vaccine. They aim here is to rescue a potent T cell immune response in chronic patients. These vaccines are to be considered as an addition to the standard therapy, and not a substitutive. A number of vaccine candidates are currently in clinical trials. Candidates that are entering phase II trials are those developed by Transgene (TG4040), Okairos (AdCh3NS3mut) and ChronTech Pharma (CHRONVAC-C).

TG4040 is based on the non-pathogenic MVA vector expressing the HCV NS3-NS5B proteins (Habersetzer, Honnet et al. 2011) and it is now in clinical phase II trial. The CHRONVAC-C DNA vaccine encoding synthetic codon-optimized HCV NS3/4A genes delivered intramuscularly with electroporation to enhance the immune response is also an interesting candidate, as it has been shown in combination with SOC to be able to reduce viral load in chronically infected HCV patients. A special safety issue has been pointed out for therapeutic vaccines against HCV since these vaccines are supposed to induce a strong HCV-specific CTL response the major concern consists in the risk of liver damage. So far none of the vaccine candidates previously described has shown any adverse side effect, but clearly none of them have yet succeeded to clear the virus completely (Feinstone, Hu et al. 2012).

4 *IN VIVO* AND *IN VITRO* MODELS FOR STUDIES OF HCV INFECTION

In this section, current available *in vitro* (cell culture) and *in vivo* (animals) models for the study of HCV infection will be discussed.

4.1 *IN VITRO* MODELS

In the early stage of HCV research field all the viral clones that were trying to propagate *in vitro* resulted to be non-functional due to the high mutation rate of the HCV RNA dependent RNA polymerase. This problem was solved by the construction of consensus genomes based on a master sequence representing the majority of viral genomes in a given sample (Boonstra, van der Laan et al. 2009). The first complementary DNA full-length functional clone of HCV was derived from a genotype 1a strain and its RNA transcripts were able to infect chimpanzees after intrahepatic inoculation (Kolykhalov, Agapov et al. 1997; Yanagi, Purcell et al. 1997). After this first consensus genome several others were made for genotypes 1 and 2 but they were all unable to replicate *in vitro* (Bartenschlager and Sparacio 2007). The first system that allowed HCV replication *in vitro* was created modifying the consensus genome Con1 by replacing the structural proteins with the neomycin resistance gene and adding a second internal ribosome entry site to promote translation of the nonstructural proteins (Lohmann, Korner et al. 1999). This so-called subgenomic replicon (or bicistronic replicon) was transfected in specific cell lines and, after selection, the cell colony in which the replicon had the highest levels of replication was isolated (Lohmann, Korner et al. 1999; Pietschmann, Lohmann et al. 2001). Highly permissive cells are the Huh7.5 and Huh7-Lunet cells (Blight, McKeating et al. 2002; Friebe, Boudet et al. 2005). These replicon systems have been used to study the HCV replication process as well as to test antiviral compounds targeting NS3 and NS5B (Bartenschlager 2005). An improvement of this system was done creating subgenomic replicons containing reporter genes like the firefly luciferase or fluorescent proteins (Lohmann, Hoffmann et al. 2003; Jones, Murray et al. 2007; Schaller, Appel et al. 2007). This system was subsequently modified with the introduction of the HLA-A2 molecules by lentiviral transduction in the Huh7-Lunet cells and the introduction of the luciferase gene in the HCV replicon directly under the control of the HCV replication machinery (Jo, Aichele et al. 2009). Monocistronic replicons have also been generated by inserting green fluorescent protein in the NS5A coding region with the purpose of discriminate between viral genomes in studies about HCV superinfection exclusion (Schaller, Appel et al. 2007). All these replication models however do not allow the secretion of viral particles and this is probably caused by adaptive mutations that are needed to enhance replication rates but on the other hand impair viral assembly (Pietschmann, Zayas et al. 2009). The situation changed when a subgenomic replicon from the JFH-1 HCV strain (genotype 2a) was constructed (Date, Miyamoto et al. 2007). Transfection of Huh7 and Huh7.5 cells with the fulllength JFH-1 genome or with a recombinant chimeric genome (combination of JFH-1 and the J6 genotype 2a isolate) resulted in the secretion of viral particles that were infectious in cultured cells and animal models (Lindenbach, Evans et al. 2005; Wakita, Pietschmann et al. 2005; Zhong, Gastaminza et al. 2005; Lindenbach,

Meuleman et al. 2006). Another important *in vitro* model for the study of early steps of virus binding and cell entry is the pseudoparticle system. This system consists in the incorporation of HCV glycoproteins E1 and E2 into retroviral or lentiviral cores that are highly infectious and that can mimic the viral entry of HCV (Bartosch, Dubuisson et al. 2003). Thanks to this model it was possible to identify several viral entry factors like glycosaminoglycan, low density lipoprotein receptor and claudin-1 (von Hahn and Rice 2008).

4.2 *IN VIVO* MODELS

Chimpanzee. Chimpanzees have so far been the best model for studies on HCV infection and related innate and adaptive host immune response. However, the HCV disease progression is somewhat different since chimpanzees more commonly clear the HCV infection spontaneously compared to humans, and the clinical manifestations are milder. Although, most of the knowledge that was acquired with chimpanzee studies would in fact not have been possible to conduct in human studies especially since the chimpanzee model allows frequent sampling of the liver for the analysis of HCV-specific T cell responses or gene expression profiles (Bukh 2004). Key aspects of HCV cellular immunity were also discovered thanks to this model as e.g. that acute resolving infection is associated with strong, intrahepatic HCV-specific CD4+ and CD8 + T cell responses (Thimme, Bukh et al. 2002; Major, Dahari et al. 2004) and that HCV persistence is associated with weaker responses and/or development of viral escape mechanisms including mutations in viral epitopes recognized by CD8+ T cells (Fuller, Shoukry et al. 2010; Callendret, Bukh et al. 2011). Furthermore, the role of the CD4+ and CD8+ T cell response was investigated in chimpanzee studies in which these cell populations have been depleted by using specific antibodies. Depletion of CD8+ T cells caused a prolonged viremia after challenge and the clearance of the virus coincided with the reappearance of these T cells in the liver (Shoukry, Grakoui et al. 2003). Depletion of CD4+ T cells instead resulted in HCV persistence after challenge indicating that an inadequate CD4+ response affects the outcome of HCV infection (Grakoui, Shoukry et al. 2003). Chimpanzees are also the only animals that can be used to evaluate the immunogenicity and efficacy of HCV vaccine candidates.

Mouse models. There are two models based on the SCID background mice; these mice lack both the T and B cells compartments. The first one is the uPA-SCID model (Mercer, Schiller et al. 2001) with mice carrying a genetic mutation that causes degeneration of hepatocytes. In this model, mice can subsequently be engrafted with primary human hepatocytes and subsequently infected with HCV. The second one is the Fah-/-Rag2-/-IL2rg-/-[FRG] model (Bissig, Wieland et al. 2010) where mice also have a genetic defect that causes liver destruction. However in this model, this is prevented by oral administration of a drug keeping the mice healthy until the engraftment with human hepatocytes. This results in a human liver repopulation of up to 95%.
Since these models are immunodeficient, they cannot be used to evaluate the adaptive immune response towards HCV. However, they have been very useful to study innate responses, virus neutralization, virus-receptor interactions and also to evaluate novel antiviral drugs. To overcome the lack of adaptive immunity in the SCID based models

two additional models have been generated. The first one is the AFC8-huHSC/hep mouse model that permits engraftment of human hepatocyte progenitor stem cells and hematopoietic stem cells so that mice will have a repopulation of the liver with human hepatocytes and an immune reconstitution with human leukocytes (Washburn, Bility et al. 2011). The other model is the Rosa26-Fluc model, which is based on an immune-competent mouse expressing human cell-surface receptors required for HCV entry (Dorner, Horwitz et al. 2011). However, the use of these models is limited to the study of only specific aspects of the HCV life cycle as HCV infection often results in undetectable viremia.

HCV transgenic mouse models Several HCV transgenic mouse model systems have been established. Currently, transgenic mice expressing HCV structural proteins or non-structural proteins e.g. the NS2, NS3, NS4A, NS4B, NS5A and NS5B either individually or in various combinations have been made. These HCV proteins are often designed to be constitutively expressed under the control of liver-specific promoters (Lerat et al 2011). Such models are useful for immunological studies of HCV. Because, if a potent HCV-specific immune response can be primed by vaccination, the transgenic mouse model would enable studies whether it has effect on the transgene in the liver. Moreover, the priming event may be further complicated by the fact that the preexisting T cells have been modulated by the persistence of HCV antigen. Since such issues are difficult to assess in the absence of an infectious small-animal model, the HCV transgenic models thus represent an important alternative.

Other small animal models. These models have generally a limited use for several reasons. In the tree shrews model (*Tupaia belangeri*), these small non rodents can be infected with HCV but with a low and variable rate (Amako, Tsukiyama-Kohara et al. 2010). Tolerized immunocompetent rats with transplanted human hepatoma cells have also been generated (Wu, Konishi et al. 2005) but they could not be used to study the cellular immune response against HCV infected cells.

5 ADOPTIVE TRANSFER OF ANTIGEN-SPECIFIC T CELLS

The adoptive transfer of antigen-specific effector cells, in particular CTL, is emerging as a promising therapeutic approach for the treatment of tumors and viral infections (Rosenberg 1999; Blattman and Greenberg 2004). One of the most promising applications is the *ex-vivo* manipulation of peripheral blood lymphocytes (PBL) from either the same patient or from a suitable donor, their clonal selection (Rosenberg, Spiess et al. 1986; Dudley, Wunderlich et al. 2002; Dudley, Wunderlich et al. 2005), or genetic manipulation (Morgan, Dudley et al. 2006) to expand the wanted antigen-specific population and the reinfusion of these cells into the recipient.

5.1 T CELL THERAPY AND HCV

The antigen-specific T cell therapy has been successfully used to treat melanoma (Rosenberg, Yannelli et al. 1994; Dudley, Wunderlich et al. 2001; Yee, Thompson et al. 2002) and viral infections such as CMV (Walter, Greenberg et al. 1995; Einsele, Roosnek et al. 2002) and EBV (Heslop, Ng et al. 1996; Bollard, Aguilar et al. 2004). In the field of HCV, so far no attempts to use antigen-specific T cells have been made even though this approach has been proposed. In particular the proposed approach is focused on the TCR gene transfer of TCRs specific for the HCV NS3 antigen (Zhang, Liu et al. 2010). Another interesting study has investigated the adoptive immunotherapy of liver allograft-derived lymphocytes treated with IL-2 and the CD3-specific mAb OKT3 in HCV-positive liver transplanted patients (Ohira, Ishiyama et al. 2009). The result of this study showed that HCV RNA titers in the sera of recipients who received the HCV -specific lymphocytes were significantly lower than the patients who did not receive these cells.

5.2 TCR GENE TRANSFER

Like for any other kind of gene transfer, the TCR gene transfer has the purpose of adding a new external gene into the recipient cell. In the case of T cells, transferring a new TCR would mean that the recipient cell would gain a new antigen specificity thus being re-directed to a new target (Dembic, Haas et al. 1986). The results of completed clinical trials (Morgan, Dudley et al. 2006; Johnson, Morgan et al. 2009) have shown that despite TCR gene therapy is possible, several important questions, especially regarding the efficacy towards the risks, still remain to be addressed. However, some important points that emerged from these studies are that for successful TCR gene therapy, the generation of high-avidity T cells is a prerequisite and that coreceptor-independent TCRs would allow the generation of both cytotoxic and helper cells to combine the antigen-specific effect (Kieback and Uckert 2010). In the following sections the main problems of TCR gene transfer are discussed and a solution is proposed.

5.2.1 Expression and correct function of exogenous TCRs

To activate a T cell and obtain an effector function, the number of TCR molecules engaged with the peptide-MHC is crucial (Viola and Lanzavecchia 1996; Hudrisier, Kessler et al. 1998). It is known that mature T cells express on their surface between 10000 to 40000 TCR molecules and this expression level directly correlates with the antigen responsiveness (Blichfeldt, Munthe et al. 1996; Schodin, Tsomides et al. 1996; Labrecque, Whitfield et al. 2001). Therefore, whether a cell presents a lower number of TCRs, this would require a higher peptide concentration to become activated. This is of particular importance when an exogenous TCR is transferred into a new T cell and will have to compete with the endogenous TCR for surface expression. Other major limiting factors to consider are the correct assembly of the exogenous TCR with the CD3z complex (as the number of CD3z is limited) (Minami, Weissman et al. 1987) and the mispairing with the endogenous TCR alpha and beta chains that can lead not only to a reduced exogenous TCR surface expression but also to a potentially dangerous new antigen specificity.
In this section a number of possible solutions to this problem are described.

Codon optimization. Allowing an optimal expression of the exogenous TCR is possible by improving the translation relevant sequences at the mRNA level. This is easily obtainable by modifying the codon usage of the mRNA structural elements (Gustafsson, Govindarajan et al. 2004). Several studies demonstrated that codon optimization improves the surface expression of exogenous TCRs in transduced cells and the tetramer binding for both human and murine TCRs (Scholten, Kramer et al. 2006; Hart, Xue et al. 2008).

RNA interference (RNAi). The aim of using RNAi would in this case be to completely shut off the expression of the endogenous TCR so that the transduced T cell will only express the exogenous TCR. In this respect, it has been shown that targeting the constant region of the endogenous TCR with a specific microRNA decreased the expression of this TCR while the exogenous one was not affected thanks to the codon optimization (Okamoto, Mineno et al. 2009).

Removal of glycosylation sites. It has been shown that removing N-glycosylation sites improves the functional avidity of transduced T cells (Kuball, Hauptrock et al. 2009).

Expression vector. The ideal vector should give the possibility to carry big inserts (i. e. alpha and beta TCR chains plus co-stimulatory molecules and selection markers) and have a high transduction rate targeting only the wanted population without the need of bulk activating the T cells. It has in fact been shown that activation induces cell division that can influence negatively the *in vivo* efficacy (Gattinoni, Finkelstein et al. 2005). Moreover, an ideal vector should express the transgene for a reasonably long time without being immunogenic for the host and, possibly, its manufacture should be cheap and easy. Unfortunately such an ideal vector is not yet available but one very interesting approach was proposed by Perro et al (Perro, Tsang et al. 2010). In this study it was found that a combination of IL-15 and IL-21 facilitated lentiviral TCR gene transfer into non-proliferating T cells and the obtained redirected T cells showed a multifunctional phenotype producing IL-2, IFN-γ and TNF-α in an antigen-specific

manner. The most common vectors used for gene therapy with their advantages and disadvantages are summarized in Table 1.

Table 1. Advantages and disadvantages of the most common vectors used for gene transfer. Modified from Mountain et al (Mountain 2000).

Vector	Advantages	Disadvantages
Adenovirus	High transfection efficiency *ex vivo* and *in vivo* Proliferating and non-proliferating targets Clinical experience acquired	Insert limit 7.5 kb Immunogenicity for the host Short duration of expression Manufacture quite difficult Safety concerns for self-replicating particles
Retrovirus	High transfection efficiency *ex vivo* Clinical experience *ex vivo* Low immunogenicity Fairly long expression	Insert limit 8 kb Low transfection efficiency *in vivo* Only proliferation targets Risk of insertional mutagenesis Manufacture extremely difficult Safety concerns for self-replicating particles
Lentivirus	Proliferating and non-proliferating targets	Insert limit 8 kb Safety concern for HIV related vectors No clinical experience Safety concerns for self-replicating particles
Adeno-associated virus (AAV)	Wide variety of targets *in vivo* Low immunogenicity Very long expression *in vivo*	Insert limit 4.5 kb Immunogenicity for the host Risk of insertional mutagenesis Little clinical experience Manufacture very difficult Safety concerns for self-replicating particles
Naked DNA	Very low immunogenicity Manufacture easy and cheap Safe profile for the host	Short duration of expression Very inefficient transfection *in vivo* and *in vitro*
Transposons	Proliferating and non-proliferation targets Good safety profile regarding insertional mutagenesis Simple manufacture	Requires transfection agents like electroporation that may affect cell viability
mRNA electroporation	Excellent for *in vitro* screening of transduced T cells	Short duration of expression
Cationic lipids	Efficient transfection *ex vivo* Low immunogenicity Good safety profile Relatively simple manufacture	Inefficient transfection *in vivo* Very short duration of expression Little clinical experience
Condensed DNA particles	Efficient transfection *ex vivo* Low immunogenicity Relatively simple manufacture	Inefficient transfection *in vivo* Very short duration of expression No clinical experience

Promote preferential pairing of exogenous TCR. In order to be functional the TCR heterodimer needs to have the same, or nearly the same, amount of TCR alpha and beta molecules inside the same cell. It is therefore desirable to use one vector encoding for both the alpha and beta TCR molecules. To ensure the same expression rate of these two molecules it would be better adding a picorna or vesicular stomatitis 2A peptide

between the two genes of interest instead of using two different promoters or an internal ribosome entry site (IRES). The virus-derived 2A linker peptide allows in fact the production of a single mRNA molecule encoding for the two genes with subsequent separation into two distinct proteins during translation. Several studies showed that the 2A mediated cleavage is close to 100% efficiency (Szymczak, Workman et al. 2004) and it is better than IRES elements for TCR expression (Leisegang, Engels et al. 2008; Peng, Cohen et al. 2009). Additional improvement to the pairing of the exogenous TCR can be achieved by the introduction of a second cysteine bond in the constant region (Cohen, Li et al. 2007; Kuball, Dossett et al. 2007). Finally, it has been demonstrated by several studies that the partial or total murinization of the exogenous TCR ensures a better surface expression in addition to avoiding the mispairing problem with the human endogenous TCR (Cohen, Zhao et al. 2006; Sommermeyer, Neudorfer et al. 2006; Voss, Kuball et al. 2006). Regarding the immunogenicity of murine TCRs, it has been demonstrated that patients treated with T cells expressing murine TCRs developed antibodies directed to the murine TCR variable region. However, this was not associated with the level of transduced cell persistence or with response to the therapy (Davis, Theoret et al. 2010).

6 AIMS

In this thesis, the possibility of generating polyfunctional HCV-specific T cell responses was explored by studies on T cell redirection and vaccination. The specific aims for each study are presented here.

Paper I:

- To generate mouse T cell clones recognizing CTL target in HCV NS3
- To characterize the functional avidity and affinity of the T cell clones
- To identify the TCRs encoded by the T cell clones
- To restore the new TCRs in human T cells by retroviral gene transfer

Paper II:

- To identify new TCRs that bind CTL target in NS5A (methods from paper I)
- To restore the new NS5A TCRs and NS3 TCRs (from paper I) in human PBL
- To test this approach with PBL from healthy donors and HCV patient
- To evaluate and compare the functional profiles and antiviral activity of TCR-redirected T cells against NS3 and NS5A

Paper III:

- To design new DNA vaccines based on the HCV NS5A immunogen used in paper II
- To compare the immunogenicity of these vaccines in pre-clinical mouse models
- To identify new T cell epitopes and evaluate functional profiles of vaccine-induced T cells in the NS5A transgenic mouse model
- To evaluate the efficiency of the vaccine-induced protection with tumor challenge in the NS5A transgenic mouse model

7 COMMENTS ON THE PAPERS' MATERIAL AND METHODS

This section is intended as a discussion on the methodology used in the hereby presented studies. The purpose is to explain the reason of choosing a particular method with advantages and limitations compared to other available techniques.

7.1 DNA VACCINATION AND ELECTROPORATION (PAPER I, II AND III)

The general aim of this thesis work was to explore the possibility to generate a specific and functional T cell immunity against HCV. The starting point of all the papers including in the thesis is the vaccination of mice (HLA-A2 transgenic in paper I and II and C57BL/6J wild-type and NS5A transgenic in paper III). In paper I and II, our goal was to generate CD8+ HCV-specific TCRs and due to the robust CTL responses previously observed with electroporation-enhanced DNA vaccination (Capone, Zampaglione et al. 2006; Ahlen, Soderholm et al. 2007), we therefore chose this approach here. The same applies to paper III for which the specific aim was to raise a functional immunity against HCV. In agreement with previous studies (Ahlen, Soderholm et al. 2007) our results show that a specific and effective CTL response against NS3 as well as NS5A could be generated by DNA vaccination followed by electroporation. The advantages of this approach over the DNA injection only are the higher DNA uptake and the immune-stimulation e.g. adjuvant effect caused by the electroporation itself by providing a local inflammation (Mathiesen 1999; Gronevik, von Steyern et al. 2005; Sallberg, Frelin et al. 2009).

7.2 HUMAN CTL EPITOPES

In paper I and II, the HCV CTL peptides used for evaluate the T cell reactivity were primarily the $NS3_{1073\text{-}1081}$ (gt1a CINGVCWTV and gt1b CVNGVCWTV) and $NS5A_{1992\text{-}2000}$ (gt1a and gt1b VLTDFKTWL) sequences. Main reason for choosing these CTL epitopes is that these are HLA-A2–restricted CTL epitopes that have been associated with spontaneous clearance of acute HCV infection (Lechner, Wong et al. 2000; Chang, Thimme et al. 2001; Urbani, Uggeri et al. 2001; Shoukry, Grakoui et al. 2003). Other peptide variants for studies of genotypic cross-reactivity of the $NS3_{1073\text{-}1081}$ epitope were also used (gt2 TISGVLWT, gt3 TIGGVMWTV, gt4 AVNGVMWTV and gt6 AINGVMWTV).

7.3 HHD ANIMAL MODELS

HLA-A2 transgenic mice, also called HHD mice (Pascolo, Bervas et al. 1997) were immunized to generate HCV-specific T cells. These mice have a human like MHC class I molecule so they can present human CTL epitopes like the one described in section 7.2. Therefore the purpose of using HHD mice was to obtain murine TCRs that are specific for human CTL epitopes. The advantage of having murine TCRs genes is mainly to avoid mispairing with the human endogenous TCRs (Cohen, Zhao et al.

2006; Sommermeyer, Neudorfer et al. 2006; Voss, Kuball et al. 2006). Regarding their use in clinical trials it was demonstrated that patients treated with T cells expressing murine TCRs developed antibodies directed to the murine TCR variable region, however this antibody response was not associated with the level of transduced cell persistence or response to the therapy (Davis, Theoret et al. 2010).

7.4 HCV -SPECIFIC T CELL HYBRID CLONES (PAPER I AND II)

HLA-A2 transgenic mice were immunized with NS3/4A or NS5A HCV DNA vaccine followed by electroporation. Purified CD8+ T cells from these mice were cultured with $NS3_{1073}$ and $NS5A_{1992}$ peptides and fused with BW TCRneg cells (BW5147 alpha-beta-cell line). This approach was chosen in order to immortalize the HCV-specific T cells for further cloning of the TCR genes from selected T cell hybrid clones. Selection was performed according to CD3 expression and to ability to secrete IL-2 and IFN-γ after stimulation with peptide loaded HHD spleenocytes (Pascolo, Bervas et al. 1997) and T2 cells. The advantages of using BW T cell hybrid clones rather than T cell clones relies on their easy culturing with no need for cytokines supplements in the medium, antigen restimulation or pre-activation. In addition, they can be used directly as reporter cells in our antigen presentation assays since they lack endogenous TCR. Therefore, this system represented a simple solution for the cloning of the TCR genes of our interest and at the same time allowed to characterize the TCR functionality for choosing the suitable TCR genes for redirection of effector cells. However, the main limitation is the lack of killing function because the CD8 molecule is not present in these hybridoma cells (Rock, Rothstein et al. 1990).

7.5 CLONING OF TCR GENES (PAPER I AND II)

To better understand the approach undertaken in our work, the next section will briefly describe the organization and rearrangement of αβ TCR genes. Here γδ TCRs will not be discussed since the hereby presented studies are exclusively focused on αβ TCRs.

7.5.1 Germ-line organization and rearrangement of mouse TCR α and β chain genes

The αβ TCR is composed of a heterodimer, which is designated the alpha (α) and beta (β) chain. Each chain is consisted of an extracellular variable (V) region and a constant (C) region as membrane-anchor. The genes encoding for the αβ TCR are expressed in cells of the T cell lineage only. Functional TCR genes are produced by rearrangements of the V and J (joining) segments in the α-chain and V, D and J in the β-chain. The α-chain DNA undergoes a Vα-Jα joining and the β-chain undergoes two joinings: Dβ-Jβ first and then Vβ-DβJβ. Transcription of the rearranged genes gives the primary transcripts that are subsequently processed to give the mRNA finally encoding for the α and β chains. The leader sequence (L) is cleaved from the nascent polypeptide chain and is not present in the final protein. There is only one Cα chain and two Cβ chains that differ of a few amino acids only.

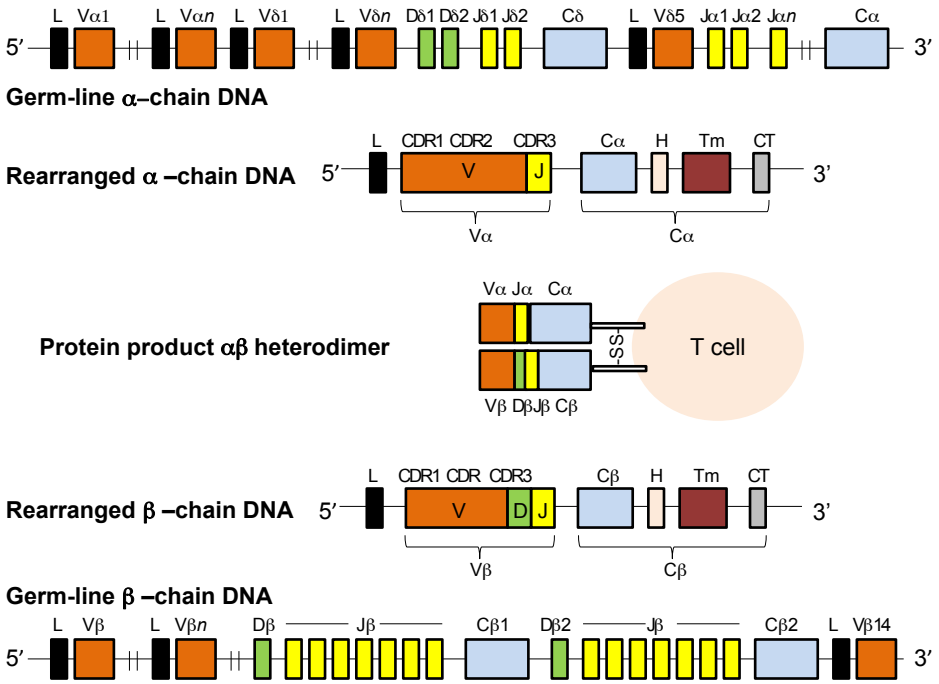

Figure 7. Example of gene rearrangement that leads to a functional αβ TCR protein heterodimer. Leader sequence (L); variable domain (V); constant domain (C); connecting sequence (H); transmembrane region (Tm); cytoplasmic tail (CT).

For the murine TCR, there are up to 75 functional alpha-chain V gene segments (Tcra-V) and around 50 for the beta-chain (Tcrb-V) (Arden, Clark et al. 1995). According to the international ImMunoGeneTics (IMGT) information system (http://www.ebi.ac.uk), these genes can be grouped in 23 possible families for the alpha-chain V genes and 31 for the beta-chain. Each family can be identified using specific primers. Several mechanisms contribute during the gene rearrangement (combinatorial joining, alternative joining, junctional flexibility, nucleotide addition etc.) to a high degree of diversity among the TCRs e.g. up to 10^{18} individual different TCRs are theoretically possible to allow recognition of pathogens. The complementary determining regions (CDR) are responsible for the binding with the MHC-peptide complex. Crystallization studies showed that the CDR3 loops of the α and β chains bind the center of the peptide; the CDR1 loop from the α-chain binds the N-term of the peptide and the CDR1 loop from the β-chain binds the C-term of the peptide; the CDR2 loops are in contact with the MHC molecule. The CDR3 loops of the α and β chains bind the center of the peptide, thus shows the highest degree of sequence variability. Most importantly, CDR3 is the main CDR responsible for recognizing the processed peptide antigen.

7.5.2 Determining the protein coding sequences of the HCV TCRs

In paper I and II, we used a multiplex PCR approach and a FACS-based antibody typing system with anti-Vβ specific antibodies to define the Vα and Vβ family usage of the identified HCV TCRs. Oligoprimers specific for each of the TCR α and β chains could then be designed and used to retrieve the full-length coding sequence of each of our HCV TCR. Although this approach could appear laborious and time consuming, one must consider that our study had to deal with screening of hundreds of

T cell hybrid clones all specific for the HCV targets of interest. Other more direct and efficient approaches, as for example the "GeneRacer" (rapid amplification of cDNA ends), which is another way to retrieve TCR genes also exists as an alternative. However, this alternative is more reasonable when dealing with a few numbers of TCR candidates.

7.6 TARGET CELLS WITH DIFFERENT HLA-A2 EXPRESSION LEVELS (PAPER I)

The function of the MHC class I molecule is to expose self-peptides mainly generated from the degradation of cytosolic endogenous proteins via the proteasome. However, cross-presentation of foreign antigens may occur when the peptides come from exogenous proteins that entered the cell for example through phagocytosis. Generally, the small peptides produced by the proteasome are released into the cytosol and subsequently translocated to the ER where they bind the MHC class I molecule. The translocation of the peptides from the cytosol into the lumen of the ER occurs via the transporter associated with antigen processing (TAP) proteins. It is in the lumen where the MHC molecule is loaded with the peptide. Normally, healthy cells display self-peptides originated during the cellular turnover, which do not activate CTLs due to central and peripheral tolerance mechanisms. But when a cell is infected by a virus and expresses viral proteins, the viral peptides will be loaded and displayed on the MHC class I molecule by which the infected cell could be recognize by the CTLs. It is for this reason that many viruses have developed immune escape strategies promoting down-regulation of the class I MHC expression on cell surface.

In our studies, we used as target cells the T2 cell line, which is commonly used as an antigen presenting cells. Being TAP deficient, the T2 cells allow easy loading of exogenous peptides on their MHC class I molecule. However one drawback of this cell model is that the density of the target molecules can be very high and thus is recognized by both high and low avidity TCRs. For this reason we also chose to test other target cell models such as the Huh-6 and C1R-A2 cell lines that have lower HLA-A2 expression to study the recognition of target cells in a more physiologic condition. For the studies performed in paper I and II, the Huh-7 replicon cells (Figure 8) were also used as a target cells. Because the Huh-7/Lunet HCV replicon cells lacked HLA-A2 expression (the endogenous HLA type are HLA-A11, -B54 and –B55 (Kurokohchi, Carrington et al. 1996; Jo, Aichele et al. 2009), these cells were engineered to express the HLA-A2 by lentiviral transduction as described by Jo et al (Jo, Aichele et al. 2009) that studied a genotype 2 HCV replicon. To better match our T cell epitopes we chose to use the HCV Con-1 strain (gt1b) replicon RNA. This model allows us to test the T cell recognition of endogenously processed $NS3_{1073}$ peptide coming from a subgenomic replicon expressing the HCV NS3-5 proteins. With this system there is a competition between the different endogenous peptides and, as a consequence, the amount of the $NS3_{1073}$ peptide presented on the HLA-A2 molecules is assumed to be lower. This model was also tested with the addition of exogenous $NS3_{1073}$ peptide.

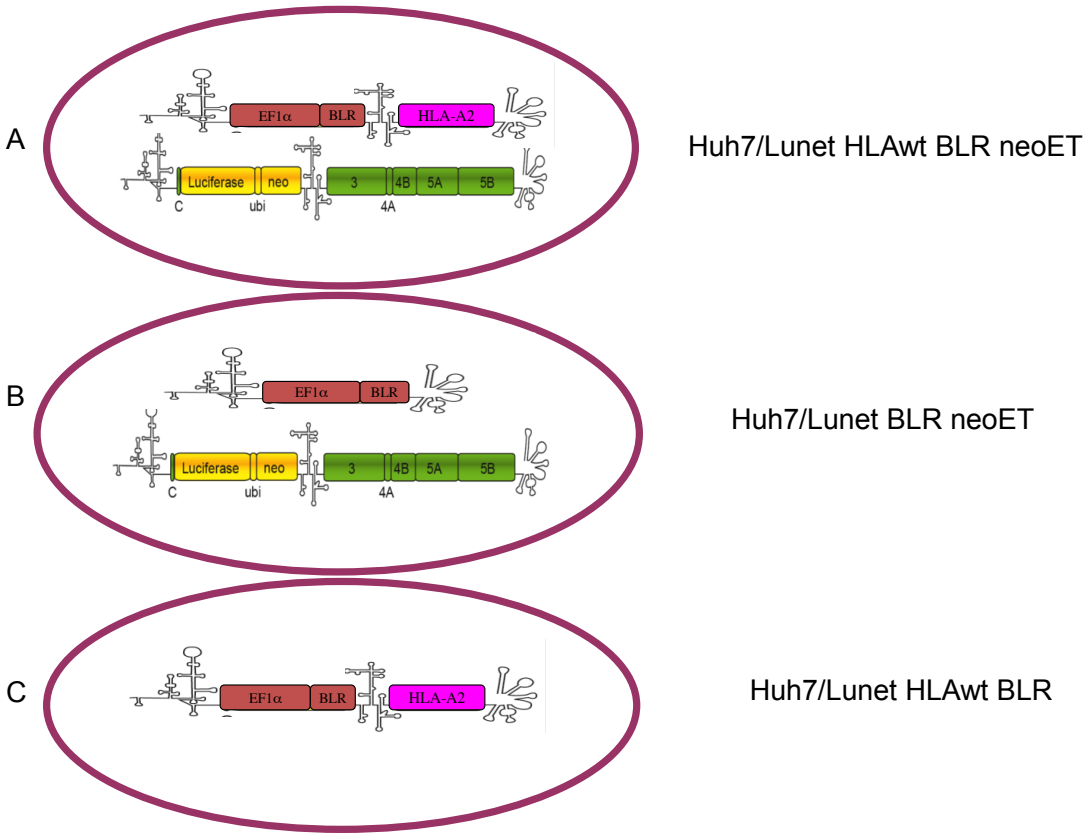

Figure 8. Schematic representation of the different Huh7/Lunet cells that are used in this thesis. A) Huh7/Lunet HLAwt BLR neoET contain the HCV replicon and the HLA-A2 gene, thus they represent the real target; B) Huh7/Lunet BLR neoET only contain the replicon, thus they represent a control for the HLA-A2 specificity; C) Huh7/Lunet HLAwt BLR only contain the HLA-A2 gene, thus they represent a control for the specificity against the HCV epitopes. Modified from Jo et al (Jo, Aichele et al. 2009).

7.7 MHC-PEPTIDE PENTAMER ANALYSIS (PAPER I, II AND III)

The MHC-peptide "pentamer" is a complex of five MHC molecules bound to a specific peptide and conjugated to a fluorocrome tag and it is primarily used to determine the frequency of antigen-specific T cells. Compared to traditional tetramers consisting of four MHC-peptide complexes that typically only allow one fluorescent label in the center of the complex, the pentamer has up to five fluorescent labels so it yields a brighter signal. Moreover MHC-peptide pentamers also have better avidity interactions with TCRs thus representing a sensible reagent for detection of antigen-specific T cells. In our studies, this technology was used for different purposes. In paper I, a $NS3_{1073}$-specific HLA-A2 pentamer was used to evaluate differences in affinity to the MHC-peptide complex within the different hybrid clones. The affinity of the TCR for the MHC-peptide complex is, in fact, usually quite weak compared for example to the antigen-antibody bond, (Kd from 10^{-4} to 10^{-7} M vs 10^{-6} to 10^{-10} M). However, the interaction between the T cell and the antigen presenting cell is strengthened by the presence of several accessory membrane molecules like CD8 or CD4, CD28, CD45R, CD86, ICAM-1 and LFA-8 and others (Oh, Hodge et al. 2003; Yang, Hodge et al.

2005). Since our T-BW hybrid clones lacked the CD8 and all the other molecules, we tested whether they could bind the $NS3_{1073}$-specific HLA-A2 pentamer. In paper II, $NS3_{1073}$- or $NS5A_{1992}$-specific HLA-A2 pentamers were used to confirm the correct pairing and surface expression of the murine TCRs transferred into human cells. Only when both α and β chains were correctly expressed and assembled, we could see pentamer-positive cells comparable to the percentage of cells stained with the TCR-specific Vβ antibody. In paper III we evaluated the frequency of NS5A-specific CD8+ T cells with a $NS5A_{2251}$-specific H-2Kb pentamer for mouse T cells. In all the studies the MHC-peptide pentamer staining was always accompanied with one or several T cell markers to increase the validity of the assay.

Variables to be considered when using antigen-specific pentamers are the following: variations of TCR density, differences in membrane lipid organization, state of T cell activation and differentiation status that can all affect the binding between the TCR and the pentamer. In view of this, one must take into account that the detected percentage of pentamer-positive cells could be an underestimation of the real number of antigen-specific cells. Another point is that it has been observed a discrepancy between pentamer binding and CTL activity especially with IFN-γ ELISpot readout (Rubio-Godoy, Dutoit et al. 2001).

7.8 PHOENIX PACKAGING CELL SYSTEM AND RETROVIRAL TRANSDUCTION OF HUMAN PBL (PAPER I AND II)

Retroviral vector-mediated gene transfer system was chosen to deliver the HCV TCR genes into T lymphocytes. We decided to use the retroviral system (the assembly of viral particles is represented in Figure 9) with a packaging cell line containing an amphotrophic envelope (Pizzato, Marlow et al. 1999) so that our recombinant particles could be made to target human T cells. Moreover, the flexibility of this system also allows us to easily package our TCR genes with other viral envelopes such as: 1) the VSV glycoprotein (VSV-G) (Frecha, Costa et al. 2008; Funke, Maisner et al. 2008) with tropism for all diving mammalian cells, or 2) the ecotrophic envelope of murine leukemia virus (MLV-E) (Albritton, Tseng et al. 1989) with tissue tropism for murine T cells. The retroviral vector chosen here is a MP71-PRE vector, that has the long terminal repeats (LTR) of the myeloproliferative sarcoma virus (MPSV) and an improved 5' untranslated leader region, designed to mediate a higher transgene expression in human and murine T cells compared to the standard Mo-MLV-based vectors (Engels, Cam et al. 2003; Leisegang, Engels et al. 2008).

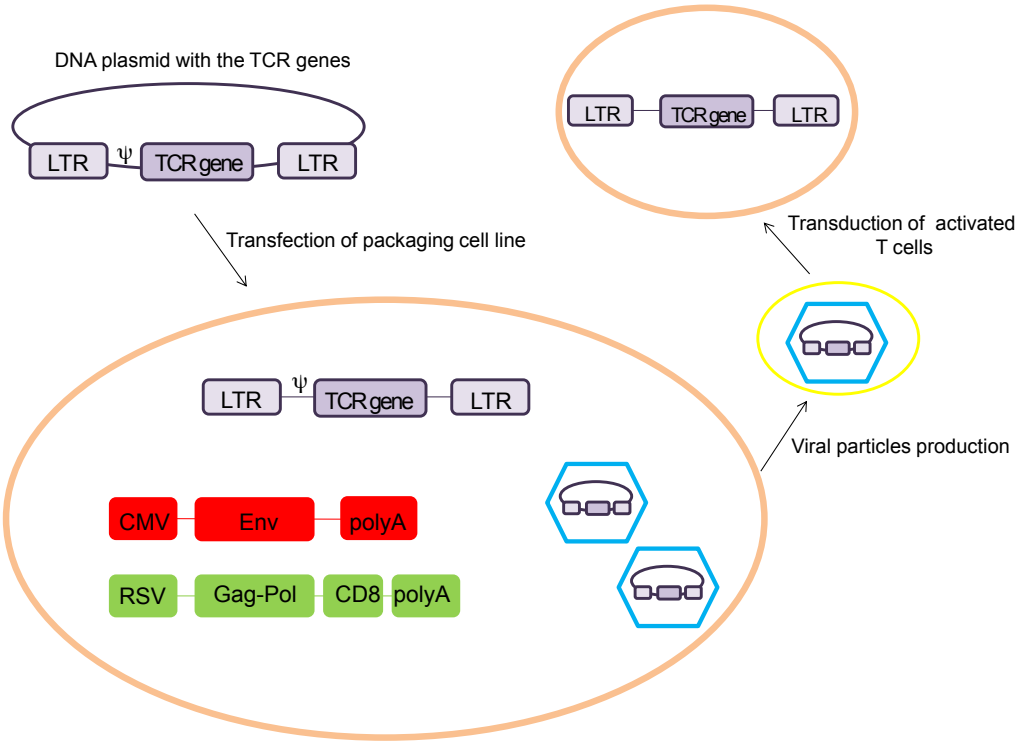

Figure 9. Phoenix packaging system (from Gary Nolan's lab, Stanford University, CA, USA). The DNA plasmid pMP71 containing the TCR genes is transfected into the packaging cell line harboring the retroviral genes. The viral particles are assembled and the TCR gene is packaged into the capsids (thanks to the ψ sequence) inside the packaging cells. The recombinant viral particles are secreted in the culture medium and can be harvested and used to transduce the target cells. Modified from http://www.stanford.edu/group/nolan/tutorials/retpkg_7_phx_sys.html

8 RESULTS AND DISCUSSION

Chronic HCV infection is one of the major causes of liver disease in the world. Despite that the introduction of new HCV-specific antiviral drugs in 2011, i.e. Telaprevir and Boceprevir, has increased the number of patients resolving the infection, not all infected individuals can be successfully cured (Jacobson, McHutchison et al. 2011; Poordad and Khungar 2011). In parallel, many vaccine candidates have been proposed but none of them is yet available in the clinic (Klade, Wedemeyer et al. 2008; Wedemeyer, Schuller et al. 2009; Eisenstein 2011; Habersetzer, Honnet et al. 2011; Torresi, Johnson et al. 2011). Several studies have tried to explain why some individuals clear the HCV infection while some others develop a chronic disease instead (Battegay, Fikes et al. 1995; Cerny, McHutchison et al. 1995; Rehermann, Chang et al. 1996; Cooper, Erickson et al. 1999; Gruner, Gerlach et al. 2000; Lechner, Wong et al. 2000; Chang, Thimme et al. 2001; Thimme, Oldach et al. 2001; Lauer, Ouchi et al. 2002; Thimme, Bukh et al. 2002; Wedemeyer, He et al. 2002; Lauer, Barnes et al. 2004; Cox, Mosbruger et al. 2005; Penna, Pilli et al. 2007). One of the current hypotheses is that acute resolving HCV infection is associated with a strong, broadly directed and sustained CD8+ T cell responses. However, a universal definition for the CD8+ T cell response in persistent HCV infection has not yet been agreed on (Neumann-Haefelin, Spangenberg et al. 2007). In general it seems that the HCV-specific CD8+ T cell response is weaker or absent in the chronic patients and that both CD4+ and CD8+ T cells display functional impairments, including reduced cytotoxicity, reduced secretion of antiviral cytokines such as IFN-γ, and a reduced proliferative capacity (Urbani, Boni et al. 2002; Wedemeyer, He et al. 2002; Spangenberg, Viazov et al. 2005). Recently it has also been shown that an effective virus clearance is associated with polyfunctional HCV-specific CD4+ and CD8+ T cells (Ciuffreda, Comte et al. 2008). Polyfunctionality has been defined by the ability of both CD4+ and CD8+ T cells to produce cytokines such as IL-2 and IFN-γ and to proliferate (Iyasere, Tilton et al. 2003; Younes, Yassine-Diab et al. 2003; Zimmerli, Harari et al. 2005). The present thesis is focused on the generation of polyfunctional T cells against HCV and in the following paragraphs the results of each study are discussed.

8.1 NOVEL HLA-A2 RESTRICTED MOUSE T-BW CELL HYBRID CLONES (PAPER I AND II)

As discussed in section 7, we generated several HCV-specific T cell hybrid clones with different antigen specificity and avidity. The main advantage of this method is the relative simplicity of the culturing conditions for the hybrid clones compared to the T cell clones. The hybrid clones can be seen as a way to immortalize an antigen-specific T cell. Moreover, hybrid clones can either be used directly in immune recognition assays as reporter cells or be considered as a stable source of specific TCRs. In paper I, we generated and characterized four T cell hybrid clones (I8H4, I8A4, I4G7 and I4F8) specific for HCV $NS3_{1073}$. The first interesting finding was that our murine TCRs exhibit similar CDR3 motifs reported for human TCRs specific to the $NS3_{1073}$ peptide (Zhang, Liu et al. 2010), as the motifs ‘GG’ and ‘FGPGTR” observed in the human

$NS3_{1073}$ TCR were also commonly found in our mouse TCRs (Table 3). They also show a type 2 TCR bias (Turner, Doherty et al. 2006), which involves the frequent selection of "motifs" in the CDR3 loop of antigen-specific TCR Vα or Vβ chains and can be limited to the consistent selection of single or multiple amino acids at specific positions in the CDR3 region (Table 2 and 3). This suggests that highly conserved CDR3 motifs are shared by murine and human TCRs specific to the $NS3_{1073}$ peptide. Only few other studies have investigated the clonality of TCRs specific for HCV (Kashii, Shimizu et al. 1997; Umemura, Yoshizawa et al. 2000) even if it seems that there is a limited usage of TCR Vβ and clonal T cell expansion in the inflammatory sites of patients with autoimmune diseases (Davies, Martin et al. 1991; Gold and Bellgrau 1991; Hoshino, Nagata et al. 1997; Arenz, Meyer zum Buschenfelde et al. 1998; Striebich, Falta et al. 1998; Yoshizawa, Ota et al. 1999). So it is possible to hypothesize a general mechanism by which a specific CDR3 motif corresponds to a certain antigen. This is reasonable if one thinks that given a certain HLA haplotype only few combinations of amino acids confer a stable binding between the TCR and the peptide-MHC complex.

Table 2. CDR3α loops of the four $NS3_{1073}$-specific murine TCRs in this thesis and the human TCR reported by Zhang et al (Zhang, Liu et al. 2010). The mouse TCRs exhibit a type II pattern bias (Turner, Doherty et al. 2006) that is characterized by the preference of the motifs 'KLTFG'. The same pattern has been found also in the CDR3α loops of the NS5A-specific murine TCRs as shown in summary Table S2.

TCR clone (α chain)	V	N	J
I8H4	CAMRE	IT	GNTGKLIFGLGTTLQVQP
I8A4	CIVTD	VE	TGGYKAVFGSGTRLLVSP
I4G7	CAVS	RD	TNTGKLTFGDGTVLTVKP
I4F8	CAVS	NM	GYKLTFGTGTSLLVDP
Human TCR (Zhang 2010)	VYYCLVGA	Y	ILTGGGNKLT

Table 3. CDR3β loops of the four $NS3_{1073}$-specific murine TCRs in this thesis and the human TCR reported by Zhang et al (Zhang, Liu et al. 2010). The motifs 'GG' and 'FGPGTR" in the human $NS3_{1073}$ TCR is also commonly found in the mouse TCRs.

TCR clone (β chain)	V	(N)D(N)	J
I8H4	CASSD	ALGGED	AEQFFGPGTRLTVL
I8A4	CAS	ASTGAS	SYEQYFGPGTRLTVL
I4G7	CASS CASS	GGLGGH DYRD	TLYFGAGTRLSVL SGNTLYFGEGSRLIVV
I4F8	CASSQ	EMGGA	EQYFGPGTRLTVL
Human TCR (Zhang 2010)	VYFCASS	TSGGAP	ETQYFGPGTR

T-BW cell hybrid clones specific for HCV $NS5A_{1992}$ were also generated with the same methodology described in paper I. After HAT selection 103 T-BW cell hybrid clones resulted CD3 positives, of these seven (named 18, 19, 37, 54, 57, 64 and 69) were also secreting IL-2 and/or IFN-γ after stimulation with peptide loaded T2. These seven clones were also tested on Huh-7/Lunet cells with and without loading of peptide and on T2 cells with two different peptide concentrations, the results are summarized in Table 4. Among the NS5A -specific T-BW cell hybrid clones the 19 and 69 were chosen as representatives of low (19) and high (69) affinity TCRs for further study of TCR redirection.

Table 4. NS5A-specific T-BW cell hybrids and their reactivity against the $NS5A_{1992}$ CTL peptide. Fold increase in cytokine production after co-culture with hepatoma (Huh-7/Lunet) or T2 target cells loaded with or without the peptide (10μg/ml or 1 μg/ml).

Clone ID	Cytokine	Huh-7/Lunet (E:T 1:1) + 10 μg/ml peptide	Huh-7/Lunet (E:T 5:1) + 10 μg/ml peptide	T2 (E:T 1:1) + 10 μg/ml peptide	T2 (E:T 1:1) + 1 μg/ml peptide
18	IL-2	0	1.5	1.5	0
	IFN-γ	0	0	0	0
19	IL-2	1.6	2.8	3.8	0
	IFN-γ	0	0	1	0
37	IL-2	0	0	1	0
	IFN-γ	0	0	1	0
54	IL-2	0	0	1	1.6
	IFN-γ	0	0	0	0
57	IL-2	2.7	5	6.7	2.9
	IFN-γ	4.3	9.3	1	0
64	IL-2	0	0	1.3	0
	IFN-γ	0	0	1	0
69	IL-2	1.6	2.8	11.4	5
	IFN-γ	2.7	4.3	1.4	1

8.2 AFFINITY TO $NS3_{1073}$/HLA-A2 PENTAMER (PAPER I)

The concept of affinity in a molecular complex (like the one formed by the TCR and the pentamer) is intended as a specific type of equilibrium (usually defined by a dissociation constant) that measures the propensity of a larger object to separate (dissociate) reversibly into smaller components (Zhou, Rivas et al. 2008). In paper I, we report that, among the four clones tested for functionality, only I8H4 was able to bind the pentamer. This in our hands represented a further confirmation that this TCR clone could be considered as of high avidity for the peptide-MHC molecule. Even if it has been shown that a good effector functionality not necessarily associates with the pentamer binding (Burrows, Kienzle et al. 2000; Hernandez, Lee et al. 2000; Rubio-Godoy, Dutoit et al. 2001; Lyons, Roszkowski et al. 2006; Laugel, van den Berg et al. 2007) it is known that the total strength of the interaction between a T cell and its target cell is determined by several factors. These are the affinity of the single TCR for the

specific peptide-MHC complex, the number of these interactions and the contribution of accessory molecules (i.e. CD4 or CD8) binding to MHC molecules upon TCR engagement (Konig 2002). One of the peculiarities of our hybrid clones, and accordingly to what has also been shown for other BW-T cell hybrid clones (Rock, Rothstein et al. 1990) is the lack of CD8 co-receptor suggesting that the I8H4 TCR must have a high avidity per se. The TCR avidity is very important *in vivo* as it seems to determine the T cell's fate (Jameson, Hogquist et al. 1995; Saito 1998). According to Valitutti et al (Valitutti, Dessing et al. 1995) there is an interval of T cell functionality between a too high avidity threshold that impairs T cell activation by reducing the ability of the TCR to dissociate from the peptide-MHC complex and a too low limit where the T cells do not receive sufficient signal for activation. On the other hand several studies have shown the importance of high avidity TCRs especially for fighting viral infections (Neveu, Debeaupuis et al. 2008) because high avidity CTL clones lyse virus infected cells earlier and more rapidly than low avidity clones at any density of antigen complexes (Derby, Alexander-Miller et al. 2001). In Valitutti et al (Valitutti, Muller et al. 1996), it was shown that different effector functions are triggered by different thresholds of antigen density and in Wiedemann et al (Wiedemann, Depoil et al. 2006) that a relatively long lived immunological synapse is required to elicit a cytokine response in CD8+ T cells whereas a short lived synapse is sufficient to effect killing. In this context, the fact that the I8H4 clone has a capability of binding the pentamer in a CD8-independent manner makes it a promising candidate for subsequent studies of TCR transfer in human cells.

8.3 COMPARISON OF THE FUNCTIONAL AVIDITY (PAPER I)

Since only one of the four selected hybrid clones was able to bind the pentamer, we could not use the off rate of the pentamer as a measure to compare the functional avidity between the clones. Therefore in our study the functional avidity was defined as the reciprocal of the antigen concentration required to elicit the half-maximal effect response, measured as IL-2 secretion (EC50). Here we compared the IL-2 release of the hybrid clones after co-culturing with T2 cells loaded with diluted amounts of $NS3_{1073}$ peptide and the result confirmed that I8A4 was a low avidity clone (400ng/ml EC50) while the other three could be considered as of moderate/high avidity (10ng/ml EC50).

8.4 CROSS-GENOTYPE REACTIVITY (PAPER I)

One of the most important features of a TCR is to be specific for its target. In our study we were interested in targeting exclusively the HCV $NS3_{1073}$ peptide. In order to test the TCR specificity in our hybrid clones, we therefore assessed their cross-reactivity to other viral peptides like the HCV $NS3_{1073}$ genotype 1b, 2, 3, 4, 5, 6, HCV $NS5_{2221}$, HCV $NS5A_{1992}$, HCMV $pp65_{495}$ and the Flu-NA_{231} peptide. This was particularly important for the Flu-NA_{231} peptide since cross-reactivity with HCV $NS3_{1073}$ genotype 1b was previously reported (Wedemeyer, Mizukoshi et al. 2001). None of the hybrid clones showed cross-reactivity to the above peptides. In view of this, we concluded that all clones were of high specificity for the genotype 1 HCV $NS3_{1073}$.

8.5 RELEVANCE FOR HYBRID CLONE REACTIVITY OF EACH AMINO ACID POSITION IN THE $NS3_{1073}$ PEPTIDE (PAPER I)

It has been proposed that one strategy of immune escape for HCV is to accumulate mutations within CTL epitopes to impair CTL recognition (Weiner, Erickson et al. 1995; Erickson, Kimura et al. 2001; Grakoui, Shoukry et al. 2003; Meyer-Olson, Shoukry et al. 2004; Timm, Lauer et al. 2004). Despite this, some epitopes, like $NS3_{1073}$, show a very low variability (Chang, Rehermann et al. 1997; Seifert, Liermann et al. 2004). The reason for this could be that mutations in this region would actually impair the viral fitness. In order to determine the key amino acid positions for the TCR and the MHC binding $NS3_{1073}$ alanine substitutes were generated and used for CTL stimulation. Results highlighted that position 2, 7 and 9 are important for the binding to the MHC molecule while positions 3, 4 and 5 are essential to the binding with the human TCR (Soderholm and Sallberg 2006). Interestingly in our study, we found that these positions do not have the same relevance in all the four murine TCRs. In particular we found that positions 3, 4, 5 and 7 are essential for all the four clones and this was expected since these are the positions essential for TCR binding, while position 2, 6, 8 and 9 are essential only for the low avidity clone I8A4.

8.6 RECOGNITION OF HCV RNA REPLICON HEPATOMA CELLS (PAPER I)

In paper I the T-BW cells hybrid clones were extensively tested on different targets. With these settings we were also able to verify whether the different target HLA-A2 expression levels could further highlight the functional differences of the hybrid clones. The Huh-7/Lunet HCV replicon model added the possibility to evaluate the responsiveness of the clones to endogenously processed antigens. Taken together the results from this experiments confirmed that the T-BW cell hybrid clones had different levels of functional avidity. In particular the I8H4 clone showed the highest avidity being the only one able to recognize the Huh-7/Lunet HCV replicon cells not loaded with exogenous peptide. In view of this, the I8H4 and I4F8 clones were chosen as the best candidates for the subsequent study on TCR redirection.

8.7 TRANSFER OF TCR GENES INTO HUMAN T CELLS (PAPER I AND II)

As previously mentioned, for clearing infectious diseases, particular attention needs to be given to high avidity CTLs. In this respect, an emerging therapeutic approach is the adoptive cell therapy with the use of redirected T cells (Duval, Schmidt et al. 2006; Rosenberg, Dudley et al. 2008; Al-Khami, Mehrotra et al. 2011). In our study, we selected the TCRs with the highest avidity for the $NS3_{1073}$ peptide (H4 and F8 described in paper I) and for the $NS5A_{1992}$ peptide (19 and 69 described in paper II) and we progressed to the TCR transfer in human T cells with the retroviral vector discussed in paper I and with the packaging system described in section 7.8. Briefly, the four TCR full-length gene pairs were codon-optimized for expression in human cells, cloned into a retroviral vector with a F2A autoprotease sequence in between the β-chain and α-chain. Recombinant viral particles were produced and activated human PBL were transduced by spinoculation (Zhang, Liu et al. 2010). The four murine TCRs all have

different Vβ usage (F8: Vβ 4, H4: Vβ 8.3, 69: Vβ 8.1–8.2, 19: Vβ 6), therefore we could use specific Vβ antibodies as markers of transgene expression in human cells. We first transduced PBL from healthy donors and tested the surface expression of the murine TCRs together with their correct αβ pairing by staining with HLA-A2 $NS3_{1073}$ and $NS5A_{1992}$-specific pentamers (as discussed in section 7.7). The transduction efficiency was 10-20% of CD3+ cells with an equal distribution on CD8+ and CD8- cells. We then assayed the peptide sensitivity on peptide-loaded T2 cells and found that NS5A TCR-transduced human T cells, as also shown by the parental T-BW cells hybrid clones, had a lower functional avidity compared to the high avidity NS3 TCR F8 and H4. With the perspective of a potential therapeutic approach, we also transduced PBL from chronic HCV patients to verify whether the TCR functionality could be restored in their T cells. Moreover our findings demonstrated that all four TCRs were correctly assembled and expressed on the cell surface by the MHC-pentamer staining.

8.8 GENERATION OF ANTIGEN-SPECIFIC POLYFUNCTIONAL T CELLS BY TCR REDIRECTION (PAPER II)

Polyfunctionality has emerged as an important T cell feature that is associated with an efficient immune response against several pathogens like HIV (Betts, Nason et al. 2006; Duvall, Precopio et al. 2008) and tuberculosis (Caccamo, Guggino et al. 2010); therefore it is desired also as result of vaccination (Aagaard, Hoang et al. 2009; Burgers, Riou et al. 2009; Abel, Tameris et al. 2010; Derrick, Yabe et al. 2011; Lang Kuhs, Ginsberg et al. 2012; Tan, Eriksson et al. 2012). In order to be defined as polyfunctional, a T cell (CD8+ or CD4+) must show at least two functions including degranulation, secretion of cytokines or chemokines (Seder, Darrah et al. 2008). To identify a protective immune response it is important that the antigen-specific T cell population secrete at the same time IFN-γ, IL-2 and TNF-α. Studies about mycobacterium tuberculosis have shown that IFN-γ has a central role in clearing the infection (Flynn, Chan et al. 1993) but it is not sufficient for protection alone (Elias, Akuffo et al. 2005). The combination of IFN-γ and TNF-α instead leaded to enhanced killing of another parasite like *Leishmania major* (Bogdan, Moll et al. 1990; Liew, Li et al. 1990). On the other hand IL-2 is essential, since it promotes T cells expansion and can also improve the CD8+ T cell memory capacity and effector functions (Williams, Holmes et al. 2006). Therefore in paper II, we analyzed the production of these three cytokines in the different TCR-redirected T cells by intracellular cytokine staining. In addition, we tested the CD107a upregulation after peptide stimulation and investigated the cytolytic activity on T2 and Huh-7/Lunet replicon target cells.

8.8.1 Multi-cytokine production

The ability of transduced cells from healthy donors and chronic HCV patients to produce TNF-α, IL-2 and IFN-γ after stimulation with $NS3_{1073}$ or $NS5A_{1992}$ peptide-loaded T2 cells was assessed in paper II. Interestingly, we found a clear difference in the number of cytokines secreted between NS3- and NS5A-redirected T cells. In particular, T cells expressing the NS3-specific TCRs H4 and F8 had up to 50% of triple and double secreting populations while the other 50% was positive for only one cytokine. On the other hand, the T cells expressing the NS5A-specific TCRs 19 and 69 had up to 60-80% of single-cytokine secreting cells (preferentially IFN-γ). . Similar

results were found in both CD8+ and CD8- populations. In Figure 10 differences in functional quality of CD8+ and CD8- TCR redirected T cells from chronic HCV patients are presented.

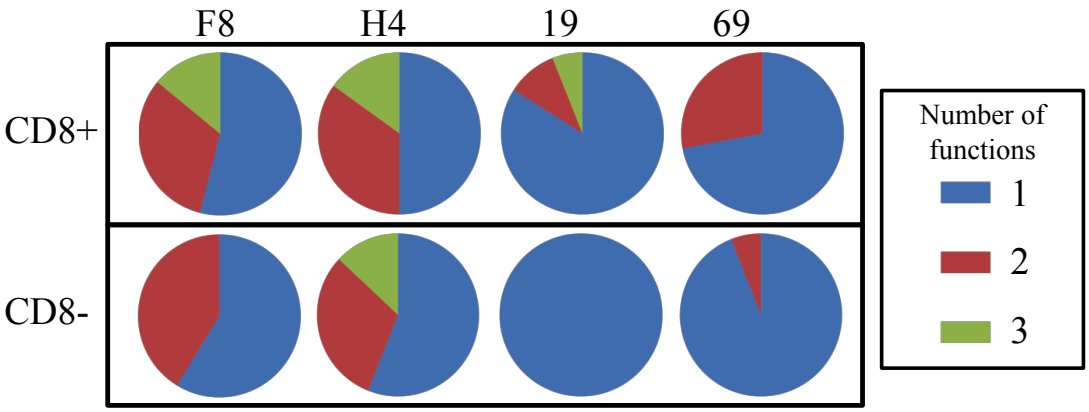

Figure 10. Differences in functional quality in TCR redirected T cells from chronic HCV patients. CD8+ and CD8- redirected T cells were assessed for the ability to secrete TNF-α, IL-2 and IFN-γ after stimulation with $NS3_{1073}$ or $NS5A_{1992}$ peptide-loaded T2.

This result is in line with the finding from a study on T cells redirected against renal cell carcinoma (RCC) cells (Leisegang, Turqueti-Neves et al. 2010). In that study, TCR from a tumor infiltrating lymphocyte, called TIL53, was isolated from a RCC (from a HLA-A2 positive patient) and cloned into the MP71-PRE retroviral vector. The constant chains of the TCR were substituted with murine constant chains and codon optimized. Human PBL from healthy donors and RCC patients retrovirally transduced with this TCR showed a multifunctional profile as demonstrated here for both our NS3-TCRs.

8.8.2 Recognition and elimination of Huh-7/Lunet replicon cells

The Huh-7/Lunet replicon cells used in this assay were engineered to express the firefly luciferase reporter protein. The advantage of this model is that since the firefly luciferase expression is controlled by the HCV replicase activity, the viral replication activity can be studied by the bioluminescence imaging system (IVIS Spectrum) using a high dimensional CCD-camera. In view of this, our approach was to culture the Huh-7/Lunet together with the redirected T cells and measure the intensity of bioluminescence. Our findings pointed out that NS3-specific redirected T cells could reduce the bioluminescence intensity of the Huh-7/Lunet replicon cells in a dose dependent manner (up to 0.01:1 E:T ratio for the H4 TCR). On the other hand, the NS5A TCR-redirected cells were less effective even if a partial reduction of bioluminescence could still be observed. With the same settings, we tested the production of IFN-γ and TNF-α and we found that H4 and F8 TCRs expressing cells were able to produce INF-γ and to some extent TNF-α while the 19 and 69 TCRs expressing cells only produced IFN-γ. This result is in accordance with the finding of Jo et al (Jo, Aichele et al. 2009), in which the antiviral mechanisms of HCV $NS5B_{2594}$ -

specific CTLs were tested using a HCV genotype 2 replicon cell system. This elegant study also demonstrated that a strong inhibition of HCV replication (up to 95%) can be observed also at low E:T ratios (up to 1:100) and it is mainly due to the action of IFN-γ.

8.8.3 CD107a surface mobilization

Surface staining of the CD107a molecule (also called LAMP-1) after stimulation with specific targets allows quantification of the recent lytic activity (Sabouri, Usuku et al. 2008). For our redirected T cells, we compared the CD107a mobilization after co-culturing with peptide-loaded T2 and Huh-7/Lunet replicon cells. We found that after the co-culture with peptide loaded T2 cells, both NS3- as well as NS5A TCRs transduced samples had an increase in CD107a surface mobilization while co-culturing with Huh-7/Lunet replicon cells caused CD107a up-regulation in the NS3 TCR-transduced T cells only. This result was particularly interesting since it may indicate that different target cells stimulate different effector functions in our redirected T cells. Our hypothesis is that the difference in antigen specificity between the TCR redirected cells reflects also a different degree of cytotoxic potential. We thus continued to examine whether the TCR-transduced cells had different effect on the Huh-7/Lunet replicon cells in terms of hepatocellular injury.

8.8.4 NS3- and NS5A TCR-redirected T cells differ in their hepatotoxic potentials

Huh-7/Lunet replicon cells were co-cultured with the TCR redirected T cells at ratio 1:1 and a kinetic analysis of the AST released in the supernatants was performed. These data were combined with the quantification of luciferase protein and with the counts and the morphology observation of the Huh-7/Lunet replicon cells from the same co-culture. All together these results showed a significant difference between the NS3 and NS5A TCR-redirected T cells in terms of cytotoxic activity, e.g. the NS3-specific effector cells are prone to induce cytolysis of Huh-7/Lunet target cells whilst the NS5A-specific cells seem to favor a non-cytotoxic mechanism to reduce the bioluminescence of the Huh-7/Lunet target cells. These findings support the observations that the non-cytolytic mechanism contributes to control HCV infection (Lohmann, Korner et al. 1999; Blight, Kolykhalov et al. 2000) and in particular IFN-γ is the main inhibitor of viral replication (Frese, Schwarzle et al. 2002); HCV-specific CD8+ T cells indeed can inhibit viral replication acting both in a direct cytolytic manner and through cytokine secretion without direct cell-to-cell contact (Liu, Zhu et al. 2003; Jo, Aichele et al. 2009). Moreover *in vivo* studies conducted with chimpanzees showed that viral clearance can occur in the absence of elevated alanine aminotransferase levels and with only minimal evidence of liver cell injury but with detectable IFN-γ messenger RNA in the liver (Bigger, Brasky et al. 2001; Su, Pezacki et al. 2002; Thimme, Bukh et al. 2002). Since the main target of HCV is the liver, an elimination of infected cells through a cytolytic manner could actually be comparable or even worse than the viral persistence in terms of liver injury. Therefore, CTL that uses an alternative effector mechanism that does not require killing of virus-infected cells might be more desirable. In the light of a possible therapeutic use of NS5A

redirected T cells it is important to consider that the non-lytic activity could be an advantage for those chronically HCV infected patients who already suffer from a severe liver damage. This approach would be intended as a complement to the antiviral therapy. Moreover, the combined effect of NS3- and NS5A-redirected T cells still needs to be evaluated *in vivo*.

8.9 GENERATION OF NS5A-SPECIFIC IMMUNE RESPONSE BY VACCINATION (PAPER III)

From the studies on TCR redirection, it has emerged that NS3- and NS5A-specific T cells are qualitatively different for many functional aspects. In particular, the NS5A-specific TCRs seem to confer an antiviral property without being cytotoxic. This could be considered as a positive aspect as HCV infected patients need to clear the infection but cannot stand a massive liver damage. Therefore the next study was focused on the characterization of the immune responses of the NS5A DNA vaccination. In this study, the immunogenicity of different domains of HCV NS5A was characterized in details in wild-type C57BL/6J mice as well in immunologically tolerant HCV NS5A-transgenic (Tg) mice.

8.9.1 Importance of NS5A domains in priming immune response

NS5A is an essential protein in the HCV replication machinery (Blight, Kolykhalov et al. 2000; Tellinghuisen, Marcotrigiano et al. 2005) and it has been proposed as a target for antiviral therapy (Coelmont, Hanoulle et al. 2010; Nettles, Gao et al. 2011; Lawitz, Gruener et al. 2012). Noticeably, it has been shown that the combined treatment of DAAs against NS3 and NS5A could obtain a sustained virological response even without the addition of interferon-alpha and ribavirin (Lok, Gardiner et al. 2012). In this study we utilized a series of DNA constructs consisting of the full-length and truncated versions of NS5A (Table 5) to characterize the vaccine properties of this protein.

Table 5. NS5A-based DNA expression plasmids used for immunogenicity evaluation.

Construct	Aminoacids in NS5A
1 (WT NS5A)	1-449 (full length)
2	1-163
3	1-215
4	1-326
5	1-378
6	31-449
7	105-449
8	211-449
9	302-449
coNS5A	1-449 (full length codon optimized)

The humoral immune response was first evaluated after DNA or protein vaccination in groups of C57BL/6J (H-2b) mice. By analyzing the NS5-specific IgG titers, it was found that the carboxyterminal domains II and III of NS5A are most important for priming NS5A-specific humoral immune responses, which is in line with that several human B cell epitopes have been found in the carboxyterminal region of NS5A (Khudyakov Yu, Khudyakova et al. 1995; Rodriguez-Lopez, Riezu-Boj et al. 1999).

8.9.2 Identification of CTL epitopes and comparison of cellular immune response in wild-type and NS5A-Tg mice

Several MHC class I epitopes for NS5A are described in other mouse haplotypes, but to our knowledge none is described for H-2b. Therefore, our primary goal here was to define these epitopes to be able to evaluate the cellular immune response to NS5A after DNA immunization. Briefly, the sequence of 87 synthetic 20-mer peptides (15 aa overlap) spanning the full-length NS5A gt1b gene and class I epitopes was predicted using the SYFPEITHI database version 1.0 (Rammensee, Bachmann et al. 1999). The peptides were synthetized and tested for stabilization of surface MHC class I expression on the RMA-S cell line. Two peptides able to bind the MHC class I molecule with high affinity were identified. In order to verify whether these two peptides corresponded to the *in vivo* processed CTL epitopes, we immunized wild-type and NS5A-Tg mice and further analyzed the specific immune response by IL-2 and IFN-γ ELISpot assay and ^{51}Cr release assay using peptide-loaded RMA-S cells. The results confirmed that the two peptides identified were able to induce IL-2 and IFN-γ in wild-type mice and IFN-γ even if to a low extent, in NS5A-Tg mice. Thus, the NS5A-Tg animals had a partially impaired T cell response to NS5A. On the other hand, the ^{51}Cr release assay pointed out that there was no difference in lytic activity between wild-type and transgenic mice. However, this could be explained by the five days *in vitro* peptide re-stimulation of spleenocytes from immunized mice. On the other hand the NS5A-Tg mice did had a significantly lower number of NS5A-specific CD8+ T cells compared to the wild-type mice when direct *ex-vivo* quantification was performed. The reason why we decided to investigate also the immune response in NS5A-Tg mice is that these are immunologically tolerized to the NS5A antigen and may better mimic the immune responses in chronic HCV patients. Here, tolerance is defined as the process by which the immune system does not react to a certain antigen. This could be a self-antigen or an external antigen. Despite in our transgenic mice the NS5A is an external antigen, its constant expression in the organism made it less reactogenic for the immune system.

8.9.3 *In vivo* protection against growth of NS5A-expressing tumor cells

To evaluate CTL functionality *in vivo* we developed a challenge model using syngeneic tumor cells (EL-4 lymphoma cells) expressing the NS5A-protein. Mice were challenged after vaccination to assess the protection efficacy against tumor growth (Encke, zu Putlitz et al. 1998; Frelin, Alheim et al. 2003). Mice were given one injection of the full-length constructs of NS5A, wild-type or codon optimized, and challenged with NS5A-EL-4. Interesting, only the mice immunized with the codon

optimized NS5A received an immunological boost by the tumor cells that express NS5A. This could be explained by the fact that the codon optimized version of NS5A primes stronger NS5A-specific immune response compared to the wild-type version. Our result also revealed that region aa 216 to 449 was important for protection against NS5A-EL-4 tumour growth. Moreover we tested tumor protection in mice vaccinated with the constructs containing truncated versions of NS5A and found that the one with intact CTL epitopes was mainly the one being able to raise a protective immune response against tumor growth, suggesting that the tumor protection observed is dependent on presence of intact CTL epitopes. Interestingly, even though the construct 9 lacks the CTL epitopes it was able to provide a tumor protection, and indicates that the protection may not only dependent on the two identified CTL epitopes. Importantly, our results suggest that the minimum region of NS5A needed to mediate protection against tumor growth and activate NS5A-specific T cell responses is the region connecting domain I and II, and the amino-terminal part of domain III. Interestingly, protection against tumor growth in immunized NS5A-Tg mice was present but was partial. This demonstrates that even in a tolerogenic environment, a T cell response can be activated by NS5A-DNA immunization and mediate tumor protection to some extent.

8.9.4 Expansion and polyfunctionality of activated NS5A-specific T cells.

The polyfunctionality of NS5A-specific T cells was evaluated after vaccination in wild-type and NS5A-transgenic mice. These two groups showed similar patterns of polycytokine secreting cells (IFN-γ, IL-2, TNF-α). Moreover, they showed similar levels of CD107a expression despite the NS5A-Tg group presented lower levels. The results suggest that NS5A-DNA immunization is an efficient method to generate a specific polyfunctional T cell response. This is particularly important because of the protective potential of this vaccine. In a study by Darrah et al (Darrah, Patel et al. 2007) a mouse model for *Leismania major* infection was used to demonstrate that protective vaccines were able to induce polyfunctional CD4+ T cells (secreting IFN-γ, IL-2, TNF-α) while non-protective vaccines induced only mono or double- cytokines secreting T cells. In our study we indeed demonstrated that NS5A DNA vaccination induces an antigen-specific polyfunctional T cell response in vaccinated wild-type and to some extent NS5A transgenic mice, which also protect from growth of HCV NS5A+ tumor cells.

9 CONCLUDING REMARKS

The hereby presented studies were focused on the discovery of new ways to improve the immunological response to a panel of HCV epitopes, which are of particular importance as having been identified in patients who cleared the infection. With this strategy, we were able to generate a polyfunctional antigen-specific T cell response using a TCR redirection approach and DNA vaccination.

In paper I, we generated four T-BW cell hybrid clones specific for HCV $NS3_{1073}$ with different grades of TCR avidity, in particular I8H4, I4G7 and I4F8 are considered as high avidity while I8A4 showed to be a low avidity clone instead. Redirect T cells expressing TCR genes from two of the high avidity clones (H4 and F8) were polyfunctional with a clear antiviral property against human hepatoma HCV replicon cells.

Paper II was focused on the comparison between TCR redirected T cells specific for $NS3_{1073}$ and $NS5A_{1992}$ peptides. In this study, only high avidity TCRs were chosen for genetic transfer. The most interesting finding was on the correlation between the antigen specificity and the antiviral activity. We demonstrated that while NS3 -specific T cells present a polyfunctional profile with a clear lytic activity against human hepatoma HCV replicon cells, NS5A-specific T cells mainly secrete IFN-γ and interfere with viral replication albeit in a less effective manner but could spare the target HCV+ hepatoma cells from cytolysis.

In paper III, we evaluated the immune response against HCV NS5A after vaccination.
In particular, and accordingly to the previously identified B cell epitopes (Khudyakov Yu, Khudyakova et al. 1995) we discovered that the C-terminus is a target for the humoral response. For what concerns the cellular response, we first identified two MHC class I epitopes restricted for $H\text{-}2K^b$ and $H\text{-}2D^b$ and then used them to evaluate the T cell response after DNA immunization in wild-type and NS5A-Tg mice. The interesting finding was that the NS5A-specific immune response could be raised in both wild-type and transgenic mice even though the second group showed evidences of tolerance (with lower levels of cytokine production after immunization). The two groups of mouse strains were also challenged with tumor cells expressing NS5A after vaccination and an effective protection against tumor growth was found. Taken together these results highlight the validity of NS5A DNA vaccination in generating a polyfunctional T cell response even in a tolerized mouse model and therefore this vaccine could be considered as a therapeutic candidate to assist in the treatment of HCV infection.

10 FUTURE PERSPECTIVES

The present thesis was proposed to investigate the possibility to generate polyfunctional effector T cells to eliminate HCV-infected cells. Our results suggest that this can be achieved; polyfunctional T cells directed against different HCV CTL targets can either be engineered *in vitro* by TCR gene transfer or raised *in vivo* by vaccination. Such effector T cells are capable to eliminate HCV RNA replication or prevent tumor growth in an antigen-specific manner. The result is encouraging since polyfunctional T cells are considered as correlates of protective antiviral immunity in chronic virus infections including HCV (Harari, Dutoit et al. 2006; Ahmed and Gottschalk 2009). However, much still remains to be explored and further investigated to make sure the approach taken here are fully effective against HCV. The next step would be to further explore these approaches to investigate their potentials *in vivo* to cure HCV infection. The TCR-reagents may also serve as tools to gain better understanding of HCV immunology. How this could be further carried out is discussed in this section.

10.1 TCR-REDIRECTED CELLS AS IMMUNE THERAPY

Despite having an impressive potential in the therapy of cancer and infectious diseases, TCR-redirected T cells may also have harmful potentials. First of all, the genetic manipulation itself, especially when delivered by integrating vectors could potentially lead to oncogenic transformation. The second concern is autoimmune reactivity e.g. when the chosen antigen is expressed in normal cells and tissues, or when a mispaired TCR becomes auto-reactive. Some of these concerns have in part already been addressed in our studies: for example, the use of mouse TCR is one way to circumvent mispairing with endogenous human TCR chains. Further improvement would be to use a non-integrating vector to decrease the risk of oncogenic transformation. Potential autoimmune reactivity usually applies to non-virus related tumor cells, in which the antigen also is expressed in normal tissue. An example is “vitiligo” caused by T cell immunotherapy of melanoma (Dudley, Wunderlich et al. 2002; Yee, Thompson et al. 2002). In view of this, it would be sensible to be able to shut down the TCR-expression or eliminating the transferred cells upon any signs of severe side effects. In this regard, administration of T cell-specific antibodies or corticosteroids to block autoimmune reactions would be useful. A more specific approach is the introduction of the herpes simplex virus thymidine kinase (HSV-TK) in the adoptive transferred T cells (Bonini, Ferrari et al. 1997; Tiberghien, Ferrand et al. 2001). However, the immunogenicity of HSV-TK, resulting in elimination of transferred gene-modified T cells, could be an issue (Riddell, Elliott et al. 1996; Berger, Flowers et al. 2006). Co-transduction of the T cells with the CD20 gene for possible depletion using the CD20-specific antibody Rituximab (Introna, Barbui et al. 2000; van Meerten, Claessen et al. 2006) is also possible; however, obvious side effect in this case, is the elimination of the patient’s B cells, which is not always desirable. An interesting new approach is the TCR editing of human T cells to completely shut off endogenous TCR expression (Provasi, Genovese et al. 2012). This technique has also been used to generate clinical-grade T cells genetically modified *ex vivo* to express a chimeric antigen receptor (CAR) to redirect the specificity to a tumor-associated antigen (Torikai, Reik et al. 2012). A future scenario for the adoptive cell therapy would be to have access to TCR-edited cell lines

specific for different HCV antigens that could be used directly in the clinic without the need for personalized *ex-vivo* modification.

Current knowledge is that T cells exist in several distinct stages of differentiation. While T cells are defined as naïve before encountering the antigen, they will develop into effector memory (T_{EM}) and long-lived central memory (T_{CM}) after activation. The latter phenotype of T cells is found to be responsible for immune surveillance and tumor eradication *in vivo* (Monteiro, Batliwalla et al. 1996; Van den Hove, Van Gool et al. 1998; Sallusto, Lenig et al. 1999; Barber, Wherry et al. 2003; Klebanoff, Gattinoni et al. 2005). Although the mechanism behind this has not been fully elucidated, several hypotheses have been proposed (Fearon, Manders et al. 2001; Kaech and Ahmed 2001; Sallusto, Geginat et al. 2004). One interesting model about T cell differentiation has been proposed by Seder et al (Seder, Darrah et al. 2008). According to this model T cell differentiation is a process where, following antigen stimulation, T cells progressively gain functionality until they reach the best polyfunctional effector status as T_{CM} and are able to secrete IL-2, IFN-γ and TNF-α. Prolonged antigen stimulation results in progressive loss of multiple cytokines secretion as well as memory potential. In Figure 10 the models for effector and memory T cell differentiation are represented.

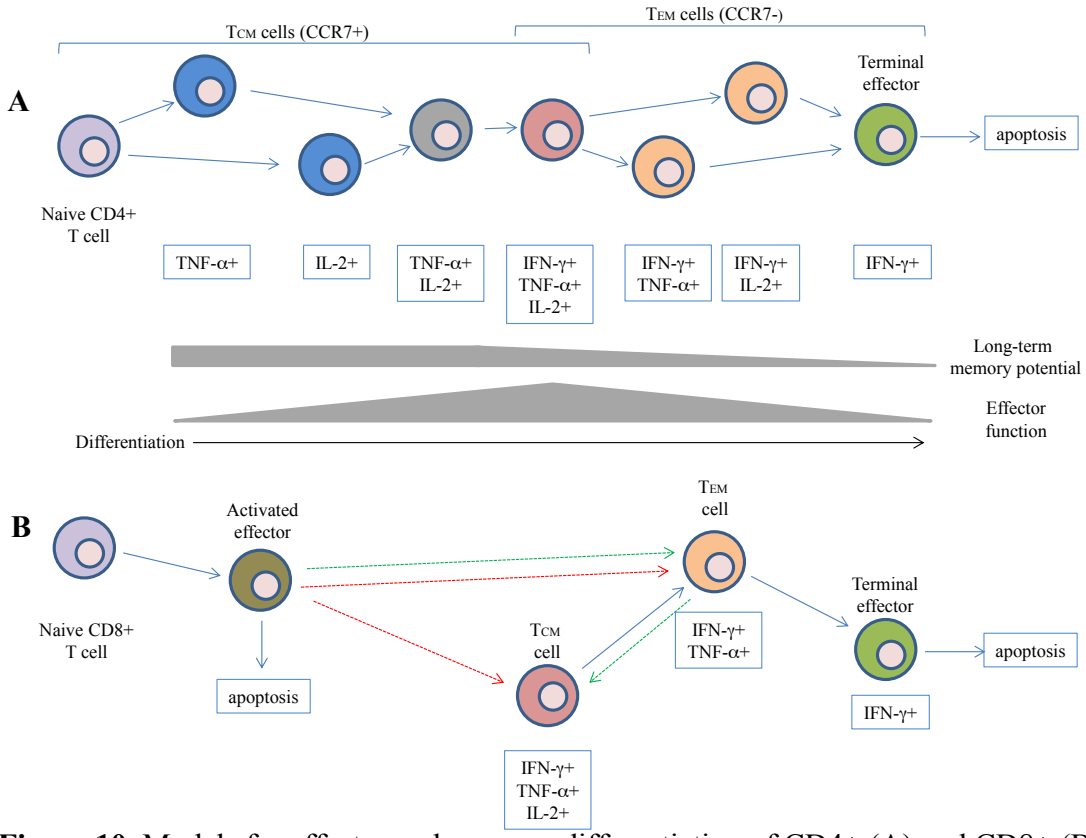

Figure 10. Models for effector and memory differentiation of CD4+ (A) and CD8+ (B) T cells. Antigen stimulation leads to the differentiation process by which the T cells gain progressively functionality until the best effector polyfunctional phenotype able to secrete IL-2, IFN-γ and TNF-α. Prolonged antigen stimulation results in progressive loss of multiple cytokines secretion as well as memory potential and eventually terminal differentiation and cell death. For CD8+ T cells two possible mechanisms for the generation of T_{CM} and T_{EM} are represented: linear differentiation (green dotted lines) and fixed lineage (red dotted lines) (Kaech and Wherry 2007). Modified from Seder et al (Seder, Darrah et al. 2008).

Taking in consideration the polyfunctional profile of T_{CM} cells it would be reasonable to use them as preferential recipient cells for our HCV TCRs to further increase their antiviral efficiency.

10.2 HOW TO TARGET THE LIVER

Being the main site of viral replication, the liver environment has mostly a suppressive effect on HCV-specific T cells. In our studies, we discussed how to generate an efficient immune response against HCV and how to direct this response to the liver to efficiently clear the virus in loco. Data obtained with transgenic mice expressing HCV antigens in the liver have demonstrated that vaccine-induced T cells can have ability to home to the liver and attack specifically the cells expressing HCV proteins (Ahlen, Soderholm et al. 2007). Studies conducted with influenza A virus also demonstrated that intranasal infection was highly efficient in inducing cytotoxic and functional CD8+ T cell responses in the liver (Keating, Yue et al. 2007; Polakos, Klein et al. 2007). Intranasal adenovirus infection also seems to be better than subcutaneous infection in inducing a functional CTL response in the liver. Therefore, the route of inoculation seems to have an importance for T cell priming (Lukens, Dolina et al. 2009), thus can have an implication in vaccination against HCV. Another interesting finding regarding the T cell homing to the liver is the discovery that the a higher level of CD161 molecule is expressed on HCV- and HBV-specific CD8+ T cells compared to CD8+ T cells specific for non-hepatotropic viruses (Northfield, Kasprowicz et al. 2008). Interestingly, the CD8+ T cells can be divided into three subpopulations depending on the CD161 staining profile: CD161-, CD161+ and CD161+++ (Takahashi, Dejbakhsh-Jones et al. 2006; Northfield, Kasprowicz et al. 2008; Billerbeck, Kang et al. 2010). The CD161+++ subpopulation in particular has an unconventional phenotype, which also reflects a unique functional profile (Klenerman and Thimme 2012). This subpopulation expresses CCR6 and CXCR6, which allow homing to the liver even under non-inflammatory conditions. CD161+ and CD161- CD8+ T cells home instead to the liver only under inflammatory conditions via other chemokine signals. A functional characteristic of these CD8+CD161+++ T cells is the ability to secrete IL-17 and IL-22, alone or in combination with IFN-γ and TNF-α (Klenerman and Thimme 2012). IL-17 may induce upregulation of other proinflammatory chemokines and cytokines on several cell types including hepatocytes, therefore in these settings, its function may be promoting inflammation and cellular recruitment (Tesmer, Lundy et al. 2008). IL-22 seems instead to have a crucial hepatoprotective role in murine models via antiapoptotic and pro-proliferative effects on hepatocytes (Wolk and Sabat 2006; Zenewicz, Yancopoulos et al. 2007). This newly identified T cell subset has thus interesting potential. A possibility in this sense may be for example the genetic modification of redirected T cells to allow different levels of CD161 expression and further evaluate their ability to specifically home to the liver.

10.3 THERAPEUTIC VACCINATION

Ideally, a therapeutic vaccine should be able to cure a chronic patient by inducing an endogenous immune repertoire in persons who are already infected. Since HCV genotype 1 virus is the most difficult to treat and the most common HCV genotype

worldwide, the effort toward a genotype 1 therapeutic vaccine, as presented in paper III, is thus justified. However, due to the high HCV mutation rate and the presence of several quasi-species, it would be sensible to develop one or more cocktails of drugs or genotype-specific vaccines. Moreover, the availability of a reliable animal model to test HCV vaccine candidates would definitely improve the results in this research area. Recently, the generation of a transgenic mouse model described by Dorner et al (Dorner, Horwitz et al. 2011) has opened new possibilities in this field and would be interesting to explore. Another aspect that must be taken into account is that the liver environment does not represent the best place for T cell priming so a vaccine that is able to efficiently prime the T cells outside this organ may have a better chance to induce an effective immune response. However the ability of the polyfunctional T cells generated after NS5A DNA immunization to migrate into the liver and clear HCV antigen expressing cells still remains to be investigated.

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